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FAO JECFA Monographs 3

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

67th meeting 2006

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Rome, 20 – 29 June, 2006

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INTRODUCTION

This volume of FAO JECFA Monographs contains specifications of identity and purity prepared at the 67th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held in Rome on 20-29 June 2006. In addition, three general analytical methods were prepared and included in this publication. The specifications monographs are one of the outputs of JECFA's risk assessment of food additives, and should be read in conjunction with the safety evaluation, reference to which is made in the section at the head of each specifications monograph. Further information on the meeting discussions can be found in the summary report of the meeting (see Annex 1), and in the full report which will be published in the WHO Technical Report series. Toxicological monographs of the substances considered at the meeting will be published in the WHO Food Additive Series.

Specifications monographs prepared by JECFA at earlier sessions, other than specifications for flavouring agents, are published in consolidated form in the Combined Compendium of Food Additive Specifications which is the first publication in the series FAO JECFA Monographs. This publication consist of four volumes, the first three of which contain the specifications monographs on the identity and purity of the food additives and the fourth volume contains the analytical methods, test procedures and laboratory solutions required and referenced in the specifications monographs. FAO maintains an on-line searchable database of JECFA specifications monographs which is available at: http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en . The database has a query page and background information in English, French, Spanish, Arabic and Chinese.

The specifications for flavouring agents evaluated by JECFA, and previously published in FAO Food and Nutrition Paper 52 and subsequent Addenda, are not included in the printed compendium, with the exception of those few which have an additional non-flavour technological function, they are included in an online searchable database at the JECFA website at FAO. http://apps3.fao.org/jecfa/flav_agents/flavag-q.jsp?language=en.

An account of the purpose and function of specifications of identity and purity, the role of JECFA specifications in the Codex system, the link between specifications and methods of analysis, and the format of specifications, are set out in the Introduction to the Combined Compendium, which is available in shortened format online on the query page, which could be consulted for further information on the role of specifications in the risk assessment of additives.

Chemical and Technical Assessment (CTAs) prepared as background documentation for the meeting will be made available online at http://www.fao.org/es/esn/jecfa/chemical_assessment_en.stm.

Contact and Feedback

More information on the work of the Committee is available from the FAO homepage of JECFA at www.fao.org/ag/agn/jecfa/index_en.stm. Readers are invited to address comments and questions on this publication and other topics related to the work of JECFA to:

jecfa@fao.org

SPECIFICATIONS FOR CERTAIN FOOD ADDITIVES

New and revised specifications

New (N) or revised (R) specifications monographs were prepared for the following food additives:

Acetylated oxidized starch (R)
Annatto extracts (alkali-processed norbixin, acid-precipitated)
Annatto extracts (alkali-processed norbixin, not acid-precipitated) (R)
Annatto extracts (aqueous-processed bixin) (R)
Annatto extracts (oil-processed bixin) (R) Tentative
Annatto extracts (solvent extracted bixin) (R)
Annatto extracts (solvent extracted norbixin) (R)
Calcium DL-malate (R)
Carob bean gum (R) Tentative
Carob bean gum (clarified) (N) Tentative
Guar gum (R) Tentative
Guar gum (clarified) (N) Tentative
Lycopene (synthetic) (N)
Lycopene from *Blakeslea trispora* (N)
DL-malic acid (R)
Maltitol (R)
Sodium hydrogen DL-malate (R)
Sodium DL-malate (R)
Titanium dioxide (R)
Zeaxanthin (synthetic) (R)

The specifications monographs are found below, with the exception of those for acetylated oxidized starch and maltitol. As regards acetylated oxidized starch, the Committee was informed of an error in the current specifications for acetylated oxidized starch, first published in the specification monograph for modified starches in the FAO Food and Nutrition Paper, 52 Addendum 9 in 2001, and republished in the Combined compendium of Food Additive specifications, FAO JECFA Monographs 1 (2005). The Committee agreed to correct the specified carboxyl value from 1.1% to 1.3%. The corrected specifications monograph for acetylated oxidized starch is included in the specifications monograph for modified starches in the JECFA on-line database for food additives (see introduction).

When the specifications for heavy metals (as lead), other metals and arsenic in sweeteners, were reviewed by the Committee at its 57th meeting in 2001, maltitol was inadvertently omitted. The Committee agreed with the Secretariat's proposal to bring the maltitol specification into line with other polyols, with regard to metals, as published in the FAO JECFA Monographs 1 (2005).

In the specifications monographs that have been assigned a tentative status, there is information on the outstanding information and a timeline by which this information should be submitted to the FAO JECFA Secretariat.

New and revised INS numbers assigned to food additives by the Codex Alimentarius Commission at its 29th session in 2006, (ALINORM 06/29/12, Appendix XVI) have been introduced in the corresponding JECFA food additive specifications monographs in the on-line database and these are not reproduced in this publication.

ANNATTO EXTRACTS (ALKALI-PROCESSED NORBIXIN, ACID-PRECIPITATED)

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI for bixin of 0 – 12 mg/kg bw and a group ADI for norbixin and its disodium and dipotassium salts of 0 – 0.6 mg/kg bw expressed as norbixin were established at the 67th JECFA (2006). The colouring matters bixin and norbixin derived from annatto extracts (solvent-extracted bixin; solvent-extracted norbixin; aqueous-processed bixin; alkali-processed norbixin, acid-precipitated; and alkali-processed norbixin, not acid-precipitated) are included in the ADIs for bixin and norbixin. All previous ADIs for annatto extracts were withdrawn.

SYNONYMS

Annatto F, Orlean, Terre orellana, L. Orange, CI (1975) 75120 (Natural Orange 4), INS 160b

DEFINITION

Alkali-processed norbixin (acid-precipitated) is prepared by removal of the outer coating of the seeds of the annatto tree (*Bixa orellana* L) with aqueous alkali. The bixin is hydrolysed to norbixin in hot alkaline solution and is acidified to precipitate the norbixin. The precipitate is filtered, dried and milled to give a granular powder.

Alkali-processed norbixin contains several coloured components; the major colouring principle is *cis*-norbixin, a minor colouring principle is *trans*-norbixin; thermal degradation products of norbixin may also be present as a result of processing.

Products supplied to the food industry may be formulated with appropriate carriers of food grade quality.

Chemical name

cis-Norbixin: 6,6'-Diapo-Ψ,Ψ-carotenedioic acid
cis-Norbixin dipotassium salt: Dipotassium 6,6'-diapo-Ψ,Ψ-carotenedioate
cis-Norbixin disodium salt: Disodium 6,6'-diapo-Ψ,Ψ-carotenedioate

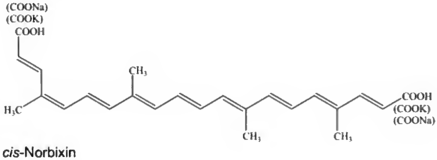
C.A.S. number

cis-Norbixin: 542-40-5
cis-Norbixin dipotassium salt: 33261-80-2
cis-Norbixin disodium salt: 33261-81-3

Chemical formula

$C_{24}H_{28}O_4$, $C_{24}H_{26}K_2O_4$, $C_{24}H_{26}Na_2O_4$

Structural formula



cis-Norbixin

Formula weight	380.5 (acid), 456.7 (dipotassium salt), 425 (disodium salt)
Assay	Not less than 15% colouring matter (expressed as norbixin)

DESCRIPTION Dark red-brown to red-purple powder

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) — Soluble in alkaline water, slightly soluble in ethanol

UV/VIS absorption (Vol. 4) — The sample in 0.5% potassium hydroxide solution shows absorbance maxima at about 453 nm and 482 nm.

Thin Layer Chromatography — Activate a TLC plate (e.g. LK6D SILICA GEL 60 A (layer thickness: 250 µm, size: 5 x 20 cm)) for 1 h at 110°. Prepare a 5% solution of the sample in 95% ethanol and apply 10 µl to the plate. Allow to dry and develop using a mixture of n-butanol, methyl ethyl ketone and 10% aqueous ammonia (3:2:2 by volume) until the solvent front has ascended about 10 cm. Allow to dry. Bixin and norbixin appear as yellow spots with R_f values of about 0.50 to 0.45, respectively. Spray with 5% sodium nitrite solution and then with 0.5 mol/l sulfuric acid and the spots immediately decolourise.

PURITY

Arsenic (Vol. 4) — Not more than 3 mg/kg
Determine using an ICP-AES/AAS-Hydride technique. Alternatively, determine arsenic using Method II of the Arsenic Limit Test. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

Lead (Vol. 4) — Not more than 2 mg/kg
Determine using an AAS ICP-AES technique appropriate to the specified level. The selection of the sample size and method of sample preparation may be based on the principles of the method described in Volume 4.

Mercury (Vol. 4) — Not more than 1 mg/kg
Determine using cold vapour atomic absorption technique. Select sample size appropriate to the specified level.

METHOD OF ASSAY Proceed as directed in Food Colours, Colouring Matters Content by Spectrophotometry (Vol. 4), procedure 1, using 0.5 % potassium hydroxide as solvent. Measure the absorbance at the A_{max} of about 482 nm. The specific absorbance ($A^{1\%}_{1\,cm}$) is 2870.

ANNATTO EXTRACTS (AQUEOUS-PROCESSED BIXIN)

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI for bixin of 0 – 12 mg/kg bw and a group ADI for norbixin and its disodium and dipotassium salts of 0 – 0.6 mg/kg bw expressed as norbixin were established at the 67th JECFA (2006). The colouring matters bixin and norbixin derived from annatto extracts (solvent-extracted bixin; solvent-extracted norbixin; aqueous-processed bixin; alkali-processed norbixin, acid-precipitated; and alkali-processed norbixin, not acid-precipitated) are included in the ADIs for bixin and norbixin. All previous ADIs for annatto extracts were withdrawn.

SYNONYMS Annatto E, Orlean, Terre orellana, L. Orange, CI (1975) 75120 (Natural Orange 4), INS 160b

DEFINITION Aqueous-processed bixin is prepared by removal of the outer coating of the seeds of the annatto tree (*Bixa orellana* L) by abrading the seeds in the presence of cold, mildly-alkaline water. The resultant preparation is acidified to precipitate bixin which is then filtered, dried and milled.

Aqueous-processed bixin contains several coloured components; the major colouring principle is *cis*-bixin, a minor colouring principle is *trans*-bixin; thermal degradation products of bixin may also be present as a result of processing.

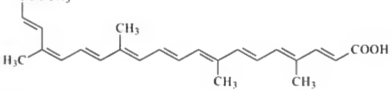
Products supplied to the food industry may be formulated with appropriate carriers of food grade quality.

Chemical name: *cis*-Bixin: Methyl (9-*cis*)-hydrogen-6,6'-diapo-Ψ,Ψ-carotenedioate

C.A.S. number: *cis*-Bixin: 6983-79-5

Chemical formula: $C_{25}H_{30}O_4$

Structural formula:



cis-Bixin

Formula weight: 394.5

Assay: Not less than 25% colouring matter (expressed as bixin)

DESCRIPTION Dark red-brown to red-purple powder

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Insoluble in water, slightly soluble in ethanol
UV/VIS absorption (Vol. 4)	The sample in acetone shows absorbance maxima at about 425, 457 and 487 nm
Thin Layer Chromatography	Activate a TLC plate (e.g. LK6D SILICA GEL 60 A (layer thickness: 250 µm, size: 5 x 20 cm)) for 1 h at 110°. Prepare a 5% solution of the sample in 95% ethanol and apply 10 µl to the plate. Allow to dry and develop using a mixture of n-butanol, methyl ethyl ketone and 10% aqueous ammonia (3:2:2 by volume) until the solvent front has ascended about 10 cm. Allow to dry. Bixin and norbixin appear as yellow spots with R_f values of about 0.50 to 0.45, respectively. Spray with 5% sodium nitrite solution and then with 0.5 mol/l sulfuric acid and the spots immediately decolourise.

PURITY

Norbixin (Vol. 4)	Not more than 7 % of total colouring matters
Arsenic (Vol. 4)	Not more than 3 mg/kg Determine using an ICP-AES/AAS-Hydride technique. Alternatively, determine arsenic using Method II of the Arsenic Limit Test. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS ICP-AES technique appropriate to the specified level. The selection of the sample size and method of sample preparation may be based on the principles of the method described in Volume 4.
Mercury (Vol. 4)	Not more than 1 mg/kg Determine using cold vapour atomic absorption technique. Select sample size appropriate to the specified level.

METHOD OF ASSAY Proceed as directed in Food Colours, Colouring Matters Content by Spectrophotometry (Vol. 4), procedure 2, using 10 ml tetrahydrofuran to dissolve the sample and acetone in place of cyclohexane. Measure the absorbance at the A_{max} of about 487 nm. The specific absorbance ($A^{1\%}_{1\,cm}$) is 3090.

ANNATTO EXTRACTS (OIL-PROCESSED BIXIN)
(TENTATIVE)

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). Due to the lack of toxicity data, no ADI was established at the 67th JECFA (2006). All previous ADIs for annatto extracts were withdrawn.

Information required on chemical characterisation of the non-colouring matter components of commercial products.

Note: The tentative specifications will be withdrawn unless the requested information is received before the end of 2008.

SYNONYMS Annatto D, Orlean, Terre orellana, L. Orange, CI (1975) 75120 (Natural Orange 4), INS 160b

DEFINITION Seeds from the annatto tree (*Bixa orellana* L) are abraded in hot vegetable oil to remove colouring matter from the surface of the seeds. The oil is sieved to remove seeds.

Oil-processed bixin contains several coloured components; the major colouring principle is *cis*-bixin, a minor colouring principle is *trans*-bixin; thermal degradation products of bixin may also be present as a result of processing.

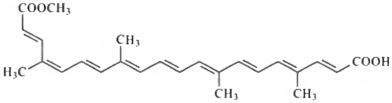
Products supplied to the food industry may be formulated with appropriate carriers of food grade quality.

Chemical name: *cis*-Bixin: Methyl (9-*cis*)-hydrogen-6,6'-diapo-Ψ,Ψ-carotenedioate

C.A.S. number: *cis*-Bixin: 6983-79-5

Chemical formula: $C_{25}H_{30}O_4$

Structural formula:



cis-Bixin

Formula weight: 394.5

Assay: Not less than 10 % colouring matter (expressed as bixin)

DESCRIPTION Dark red-brown to red-purple oil

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Insoluble in water, slightly soluble in ethanol
UV/VIS absorption (Vol. 4)	The sample in acetone shows absorbance maxima at about 425, 457 and 487 nm
Thin Layer Chromatography	Activate a TLC plate (e.g. LK6D SILICA GEL 60 A (layer thickness: 250 μm, size: 5 x 20 cm)) for 1 h at 110°. Prepare a 5% solution of the sample in 95% ethanol and apply 10 μl to the plate. Allow to dry and develop using a mixture of n-butanol, methyl ethyl ketone and 10% aqueous ammonia (3:2:2 by volume) until the solvent front has ascended about 10 cm. Allow to dry. Bixin and norbixin appear as yellow spots with R_f values of about 0.50 to 0.45, respectively. Spray with 5% sodium nitrite solution and then with 0.5 mol/l sulfuric acid and the spots immediately decolourise.

PURITY

Arsenic (Vol. 4)	Not more than 3 mg/kg Determine using an ICP-AES/AAS-Hydride technique. Alternatively, determine arsenic using Method II of the Arsenic Limit Test. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS ICP-AES technique appropriate to the specified level. The selection of the sample size and method of sample preparation may be based on the principles of the method described in Volume 4.
Mercury (Vol. 4)	Not more than 1 mg/kg Determine using cold vapour atomic absorption technique. Select sample size appropriate to the specified level.

METHOD OF ASSAY Proceed as directed in Food Colours, Colouring Matters Content by Spectrophotometry (Vol. 4), procedure 2, using 10 ml tetrahydrofuran to dissolve the sample and acetone in place of cyclohexane. Measure the absorbance at the A_{max} of about 487 nm. The specific absorbance ($A^{1\%}_{1\,cm}$) is 3090.

ANNATTO EXTRACTS (SOLVENT-EXTRACTED BIXIN)

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI for bixin of 0 – 12 mg/kg bw and a group ADI for norbixin and its disodium and dipotassium salts of 0 – 0.6 mg/kg bw expressed as norbixin were established at the 67th JECFA (2006). The colouring matters bixin and norbixin derived from annatto extracts (solvent-extracted bixin; solvent-extracted norbixin; aqueous-processed bixin; alkali-processed norbixin, acid-precipitated; and alkali-processed norbixin, not acid-precipitated) are included in the ADIs for bixin and norbixin. All previous ADIs for annatto extracts were withdrawn.

SYNONYMS

Annatto B, Orlean, Terre orellana, L. Orange, CI (1975) 75120 (Natural Orange 4), INS 160b

DEFINITION

Solvent-extracted bixin is obtained by the removal of the outer coating of the seeds of the annatto tree (*Bixa orellana* L) with one or more of the following food grade solvents: acetone, methanol, hexane, ethanol, isopropyl alcohol, ethyl acetate, alkaline alcohol or carbon dioxide. The resulting preparation may be acidified, followed by the removal of the solvent, drying and milling.

Solvent-extracted bixin contains several coloured components; the major colouring principle is *cis*-bixin, a minor colouring principle is *trans*-bixin; thermal degradation products of bixin may also be present as a result of processing.

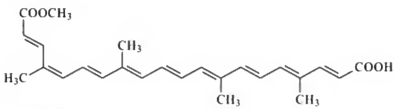
Products supplied to the food industry may be formulated with appropriate carriers of food grade quality.

Chemical name: *cis*-Bixin: Methyl (9-*cis*)-hydrogen-6,6'-diapo-Ψ,Ψ-carotenedioate

C.A.S. number: *cis*-Bixin: 6983-79-5

Chemical formula: $C_{25}H_{30}O_4$

Structural formula:



cis-Bixin

Formula weight: 394.5

Assay: Not less than 85 % colouring matter (expressed as bixin)

DESCRIPTION

Dark red-brown to red-purple powder

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Insoluble in water, slightly soluble in ethanol
UV/VIS absorption (Vol. 4)	The sample in acetone shows absorbance maxima at about 425, 457 and 487 nm
Thin Layer Chromatography	Activate a TLC plate (e.g. LK6D SILICA GEL 60 A (layer thickness: 250 µm, size: 5 x 20 cm)) for 1 h at 110˚. Prepare a 5% solution of the sample in 95% ethanol and apply 10 µl to the plate. Allow to dry and develop using a mixture of n-butanol, methyl ethyl ketone and 10% aqueous ammonia (3:2:2 by volume) until the solvent front has ascended about 10 cm. Allow to dry. Bixin and norbixin appear as yellow spots with R_f values of about 0.50 to 0.45, respectively. Spray with 5% sodium nitrite solution and then with 0.5 mol/l sulfuric acid and the spots immediately decolourise.

PURITY

Residual solvents (Vol. 4)	Acetone:	Not more than 30 mg/kg
	Methanol:	Not more than 50 mg/kg
	Hexane:	Not more than 25 mg/kg
	Ethanol: Isopropyl alcohol: Ethyl acetate:	Not more than 50 mg/kg, singly or in combination

Norbixin (Vol. 4)	Not more than 2.5 % of total colouring matters
Arsenic (Vol. 4)	Not more than 3 mg/kg Determine using an ICP-AES/AAS-Hydride technique. Alternatively, determine arsenic using Method II of the Arsenic Limit Test. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS ICP-AES technique appropriate to the specified level. The selection of the sample size and method of sample preparation may be based on the principles of the method described in Volume 4.
Mercury (Vol. 4)	Not more than 1 mg/kg Determine using cold vapour atomic absorption technique. Select sample size appropriate to the specified level.

METHOD OF ASSAY Proceed as directed in Food Colours, Colouring Matters Content by Spectrophotometry (Vol. 4), procedure 2, using 10 ml tetrahydrofuran to dissolve the sample and acetone in place of cyclohexane. Measure the absorbance at the A_{max} of about 487 nm. The specific absorbance ($A^{1\%}_{1\,cm}$) is 3090.

CALCIUM DL-MALATE

Prepared at the 67th JECFA (2006), published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 27th JECFA (1983) and published in FNP 52 (1992) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). Metals and arsenic specifications were revised at the 59th JECFA (2002). A group ADI 'not specified' for malic acid and its Ca, K and Na salts was established at the 23rd JECFA (1979).

SYNONYMS DL-Monocalcium malate; INS No. 352(ii)

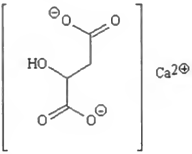
DEFINITION

Chemical names: Monocalcium DL-malate, 2-hydroxybutanedioic acid monocalcium salt

C.A.S. number: 17482-42-7

Chemical formula: $C_4H_4CaO_5$

Structural formula:



Formula weight: 172.1

Assay: Not less than 97.5% after drying

DESCRIPTION White, colourless powder

FUNCTIONAL USES Buffering agent

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4): Slightly soluble in water, insoluble in ethanol

Test for malate (Vol. 4): Passes test
Test 100 ml of a saturated solution of the sample

Test for calcium (Vol. 4): Passes test

PURITY

Loss on drying (Vol. 4)	Not more than 2% (110°, 3 h)
Fluoride (Vol. 4)	Not more than 30 mg/kg (Method III)
Fumaric acid and maleic acid (Vol. 4)	Not more than 1.0% of fumaric acid and not more than 0.05% of maleic acid
Lead (Vol.4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

METHOD OF ASSAY Weigh accurately about 0.4 g of the sample, previously dried at 110° for 3 h, dissolve in a mixture of 10 ml of water and 2 ml of dilute hydrochloric acid TS, and dilute to about 100 ml with water. While stirring (preferably with a magnetic stirrer) add about 30 ml of 0.05 M disodium ethylenediaminetetra- acetate from a 50-ml buret, then add 15 ml of sodium hydroxide TS and 300 mg of hydroxynaphtol blue indicator, and continue the titration to a blue end-point. Each ml of 0.05 M disodium ethylenediaminetetraacetate is equivalent to 8.607 mg of $C_4H_4CaO_5$.

CAROB BEAN GUM
(TENTATIVE)

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7. (1999) and in the Combined Compendium of Food Additive Specifications, FAO JECFA monographs 1. An ADI "not specified" was established at the 25th JECFA (1981).

Information required on gum content, solubility in water and a test method to determine ethanol and isopropanol using capillary gas chromatography.

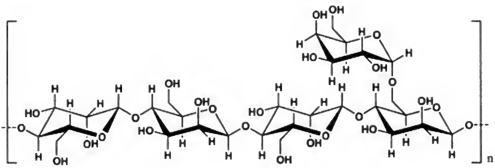
Note: The tentative specifications will be withdrawn unless the required information is received before the end of 2007.

SYNONYMS INS No. 410

DEFINITION Primarily the ground endosperm of the seeds from *Ceratonia siliqua* (L.) Taub. (Fam. *Leguminosae*) mainly consisting of high molecular weight (approximately 50,000-3,000,000) polysaccharides composed of galactomannans; mannose:galactose ratio is about 4:1. The seeds are dehusked by treating the kernels with dilute sulfuric acid or with thermal mechanical treatments, followed by milling and screening of the peeled seeds to obtain the endosperm (native carob bean gum). The gum may be washed with ethanol or isopropanol to control the microbiological load (washed carob bean gum).

C.A.S. number 9000-40-2

Structural formula



DESCRIPTION White to yellowish white, nearly odourless powder

FUNCTIONAL USES Thickener, stabilizer, emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in ethanol

Gel formation	Add small amounts of sodium borate TS to an aqueous dispersion of the sample; a gel is formed.
Viscosity	Transfer 2 g of the sample into a 400-ml beaker and moisten thoroughly with about 4 ml of isopropanol. Add 200 ml of water with vigorous stirring until the gum is completely and uniformly dispersed. An opalescent, slightly viscous solution is formed. Transfer 100 ml of this solution into another 400-ml beaker. Heat the mixture in a boiling water bath for about 10 min and cool to room temperature. There is no appreciable increase in viscosity.
Gum constituents (Vol. 4)	Proceed as directed under Gum Constituents Identification using 100 mg of the sample instead of 200 mg and 1 - 10 µl of the hydrolysate instead of 1 - 5 µl. Use galactose and mannose as reference standards. These constituents should be present.
Microscopic examination	Disperse a sample of the gum in an aqueous solution containing 0.5% iodine and 1% potassium iodide on a glass slide and examine under a microscope. Carob bean gum contains long stretched tubiform cells, separated or slightly interspaced. Their brown contents are much less regularly formed than in Guar gum.

PURITY

Loss on drying (Vol. 4)	Not more than 14.0% (105°, 5 h)
Total ash (Vol. 4)	Not more than 1.5%
Acid-insoluble matter (Vol. 4)	Not more than 4.0%
Protein (Vol. 4)	Not more than 7.0% Proceed as directed under nitrogen determination (Kjeldahl method). the percentage of nitrogen determined multiplied by 6.25 gives the percent protein in the sample
Starch	To a 1 in 10 dispersion of the sample add a few drops of iodine TS; no blue colour is produced
Ethanol and isopropanol	Not more than 1%, singly or in combination See description under TESTS
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.
Microbiological criteria	Total plate count (Vol. 4): Not more than 5,000 CFU/g *E. coli:* Negative in 1g See description under TESTS *Salmonella* (Vol. 4): Negative in 25g Yeasts and moulds (Vol. 4): Not more than 500 CFU/g

TESTS

PURITY TESTS

Ethanol and isopropanol

Information required on a method using capillary gas chromatography to replace the method below.

Principle
The alcohols are converted to the corresponding nitrite esters and determined by *headspace gas chromatography.*

Sample preparation
Disperse 100 mg of sample in 10 ml of water using sodium chloride as a dispersing agent if necessary.

Internal standard solution
Prepare an aqueous solution containing 50 mg/l of n-propanol.

Standard alcohol solution
Prepare an aqueous solution containing 50 mg/l each of ethanol and isopropanol.

Procedure
Weigh 200 mg of urea into a 25-ml "dark vial" (Reacti-flasks, Pierce, Rockford, IL, USA, or equivalent). Purge with nitrogen for 5 min and then add 1 ml of saturated oxalic acid solution, close with a rubber stopper and swirl. Add 1 ml of sample dispersion, 1 ml of internal standard solution, and simultaneously start a stop watch (T=0). Swirl the vial and recap with an open screw cap fitted with a silicone rubber septum. Swirl until T=30 sec. At T=45 sec inject through the septum 0.5 ml of an aqueous solution of sodium nitrite (250 g/l). Swirl until T=70 sec and at T=150 sec withdraw through the septum 1 ml of the headspace using a pressure lock syringe (Precision Sampling Corp., Baton Rouge, Louisiana, USA, or equivalent).

Gas chromatography
Insert syringe needle in the injection port; precompress the sample, then open the syringe and inject the sample.
Use the following conditions:
- Column: glass (4mm i.d., 90 cm)
- Packing: first 15 cm packed with chrompack (or equivalent) and the remainder with Porapak R 120-150 mesh (or equivalent)
- Carrier gas: nitrogen (flow rate: 80 ml/min)
- Detector: flame ionization
- Temperatures: injection port: 250°; column: 150° isothermal

Calculation
Quantify the ethanol and isopropanol present in the sample by comparing the peak areas with the corresponding peaks obtained by chromatographing the headspace produced by substituting in the procedure 1 ml of Standard alcohol solution for 1 ml of sample solution.

Microbiological criteria

E. coli determination

the gum sample prior to analysis is essential in order to avoid gelling of the gum during its addition to the enrichment broth. Prepare a 1.0% mannosidase solution (1 g mannan endo-1,4-betamannosidase to 99 ml water) and sterilize by filtration through a 0.45 µm membrane. (The mannosidase solution may be stored at 2-5° for up to two weeks.)
Into a sterile tube containing 9 ml of sterile lauryl sulfate tryptose (LST) broth, aseptically add 0.1 ml of the sterile 1% mannosidase solution. Add 1g gum sample to the tube and vortex vigorously to disperse the sample. Incubate the tube for 24-48 h at 35±1°. After 24 h, gently agitate the tube and examine for gas production, i.e. effervescence. Reincubate for an additional 24 hours if no gas evolution is observed. Examine a second time for gas. Perform the confirmation test for coliforms on the presumptive positive (gassing) result, according to the procedure in Volume 4.

GUAR GUM
(TENTATIVE)

Prepared tentative at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 53rd JECFA (1999) and published in FNP 52 Add 7 (1999) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI "not specified" was established at the 19th JECFA (1975).

Information required on gum content, solubility in water and a test method to determine ethanol and isopropanol using capillary gas chromatography.

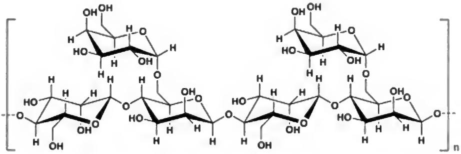
Note: The tentative specifications will be withdrawn unless the required information is received before the end of 2007.

SYNONYMS Gum cyamopsis, guar flour; INS No. 412

DEFINITION Primarily the ground endosperm of the seeds from *Cyamopsis tetragonolobus* (L.) Taub. (Fam. *Leguminosae*) mainly consisting of high molecular weight (50,000-8,000,000) polysaccharides composed of galactomannans; mannose:galactose ratio is about 2:1. The seeds are dehusked, milled and screened to obtain the ground endosperm (native guar gum). The gum may be washed with ethanol or isopropanol to control the microbiological load (washed guar gum).

C.A.S. number 9000-30-0

Structural formula



DESCRIPTION White to yellowish-white, nearly odourless, free-flowing powder

FUNCTIONAL USES Thickener, stabilizer, emulsifier

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in ethanol

Gel formation	Add small amounts of sodium borate TS to an aqueous dispersion of the sample; a gel is formed.
Viscosity	Transfer 2 g of the sample into a 400-ml beaker and moisten thoroughly with about 4 ml of isopropanol. Add 200 ml of water with vigorous stirring until the gum is completely and uniformly dispersed. An opalescent, viscous solution is formed. Transfer 100 ml of this solution into another 400-ml beaker, heat the mixture in a boiling water bath for about 10 min and cool to room temperature. There is no substantial increase in viscosity.
Gum constituents (Vol. 4)	Proceed as directed under Gum Constituents Identification using 100 mg of the sample instead of 200 mg and 1 - 10 µl of the hydrolysate instead of 1 - 5 µl. Use galactose and mannose as reference standards. These constituents should be present.
Microscopic examination	Place some ground sample in an aqueous solution containing 0.5% iodine and 1% potassium iodide on a glass slide and examine under a microscope. Guar gum shows close groups of round to pear formed cells, their contents being yellow to brown.

PURITY

Loss on drying (Vol. 4)	Not more than 15.0% (105°, 5 h)
Borate	Disperse 1 g of the sample in 100 ml of water. The dispersion should remain fluid and not form a gel on standing. Mix 10 ml of dilute hydrochloric acid with the dispersion, and apply one drop of the resulting mixture to turmeric paper. No brownish red colour is formed, which upon drying becomes intensified and changes to greenish black when moistened with ammonia TS.
Total ash (Vol. 4)	Not more than 1.5%
Acid insoluble matter (Vol. 4)	Not more than 7.0%
Protein (Vol. 4)	Not more than 10.0% Proceed as directed under Nitrogen Determination (Kjeldahl Method). The percent of nitrogen in the sample multiplied by 6.25 gives the percent of protein in the sample.
Ethanol and isopropanol	Not more than 1%, singly or in combination See description under TESTS
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

Microbiological criteria

Total plate count (Vol. 4): Not more than 5,000 CFU/g

E. coli: Negative in 1g
See description under TESTS

Salmonella (Vol. 4): Negative in 25g

Yeasts and moulds (Vol. 4): Not more than 500 CFU/g

TESTS

PURITY TESTS

Ethanol and isopropanol

Information required on a method using capillary gas chromatography to replace the method below.

Principle
The alcohols are converted to the corresponding nitrite esters and determined by *headspace gas chromatography.*

Sample preparation
Disperse 100 mg of sample in 10 ml of water using sodium chloride as a dispersing agent if necessary.

Internal standard solution
Prepare an aqueous solution containing 50 mg/l of n-propanol.

Standard alcohol solution
Prepare an aqueous solution containing 50 mg/l each of ethanol and isopropanol.

Procedure
Weigh 200 mg of urea into a 25-ml "dark vial" (Reacti-flasks, Pierce, Rockford, IL, USA, or equivalent). Purge with nitrogen for 5 min and then add 1 ml of saturated oxalic acid solution, close with a rubber stopper and swirl. Add 1 ml of sample dispersion, 1 ml of internal standard solution, and simultaneously start a stop watch (T=0). Swirl the vial and recap with an open screw cap fitted with a silicone rubber septum. Swirl until T=30 sec. At T=45 sec inject through the septum 0.5 ml of an aqueous solution of sodium nitrite (250 g/l). Swirl until T=70 sec and at T=150 sec withdraw through the septum 1 ml of the headspace using a pressure lock syringe (Precision Sampling Corp., Baton Rouge, Louisiana, USA, or equivalent.

Gas chromatography
Insert syringe needle in the injection port; precompress the sample, then open the syringe and inject the sample.
Use the following conditions:
- Column: glass (4mm i.d., 90 cm)
- Packing: first 15 cm packed with chrompack (or equivalent) and the remainder with Porapak R 120-150 mesh (or equivalent)
- Carrier gas: nitrogen (flow rate: 80 ml/min)
- Detector: flame ionization
- Temperatures: injection port: 250°; column: 150° isothermal

Calculation

Quantify the ethanol and isopropanol present in the sample by comparing the peak areas with the corresponding peaks obtained by chromatographing the headspace produced by substituting in the procedure 1 ml of Standard alcohol solution for 1 ml of sample solution.

Microbiological criteria

E. coli determination

The use of mannan endo-1,4-betamannosidase (EC 3.2.1.78) to degrade the gum sample prior to analysis is essential in order to avoid gelling of the gum during its addition to the enrichment broth. Prepare a 1.0% mannosidase solution (1 g mannan endo-1,4-betamannosidase to 99 ml water) and sterilize by filtration through a 0.45 µm membrane. (The mannosidase solution may be stored at 2-5° for up to two weeks.) Into a sterile tube containing 9 ml of sterile lauryl sulfate tryptose (LST) broth, aseptically add 0.1 ml of the sterile 1% mannosidase solution. Add 1g gum sample to the tube and vortex vigorously to disperse the sample. Incubate the tube for 24-48 h at 35±1°. After 24 h, gently agitate the tube and examine for gas production, i.e. effervescence. Reincubate for an additional 24 hours if no gas evolution is observed. Examine a second time for gas. Perform the confirmation test for coliforms on the presumptive positive (gassing) result, according to the procedure in Volume 4.

LYCOPENE (SYNTHETIC)

New specifications prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006). A group ADI of 0-0.5 mg/kg bw for synthetic lycopene and lycopene from Blakeslea trispora was established at the 67th JECFA (2006).

SYNONYMS INS 160d

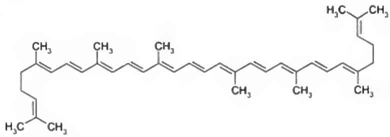
DEFINITION Synthetic lycopene is produced by the Wittig condensation of synthetic intermediates commonly used in the production of other carotenoids used in food. Synthetic lycopene consists predominantly of all-*trans*-lycopene together with 5-*cis*-lycopene and minor quantities of other isomers. Commercial lycopene preparations intended for use in food are formulated as suspensions in edible oils or water-dispersible powders and are stabilised with antioxidants.

Chemical names
Ψ,Ψ-carotene
all-*trans*-lycopene
(all-E)-lycopene
(all-E)-2,6,10,14,19,23,27,31-octamethyl-2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene

C.A.S. number 502-65-8

Chemical formula $C_{40}H_{56}$

Structural formula



Formula weight 536.9

Assay Not less than 96% total lycopenes; not less than 70% all-*trans*-lycopene

DESCRIPTION Red crystalline powder

FUNCTIONAL USES Colour, nutrient supplement

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Insoluble in water, freely soluble in chloroform

Test for carotenoids The colour of the solution of the sample in acetone disappears after successive additions of a 5% solution of sodium nitrate and 1N

	sulfuric acid
Solution in chloroform	A 1% solution is clear and has intensive red-orange colour
Spectrophotometry (Vol. 4)	A solution in hexane shows an absorption maximum at approximately 470 nm

PURITY

Loss on drying (Vol. 4)	Not more than 0.5% (40°, 4 h at 10 mm Hg)
Lead (Vol. 4)	Not more than 1 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.
Apo-12'-lycopenal	Not more than 0.15% See description under TESTS
Triphenyl phosphine oxide (TPPO) (Vol. 4)	Not more than 0.01%

TESTS

PURITY TESTS

Apo-12'-lycopenal

Determine by HPLC using the following conditions:

Reagents (Note: all solvents should be HPLC-grade):
Hexane
Triethylamine (TEA)
Tetrahydrofuran (THF)
Toluene stabilised with BHT (0.5 g BHT in 1000 ml toluene)
Apo-12'-lycopenal (also known as lycopene C_{25}-aldehyde) standard (available from DSM Nutritional Products)

Apparatus:
HPLC system with a suitable pump, injector, and integrator

Column:	Stainless steel (200x4.0 mm)
Stationary phase:	Nucleosil Si 100 3 µm (Macherey-Nagel or equivalent)
Detector:	UV/VIS or VIS

HPLC conditions:

Flow:	2.0 ml/min
Injection volume:	5.0 µl
Pressure:	approx. 135 bar
Detection:	435 nm
Mobile phase:	A – hexane B – Hexane:TEA (99.9:0.1) (v/v) C – Hexane:THF (80:20) (v/v)

Gradient:

Time, min	A%	B%	C%
0	80	20	0
16	60	20	20
22	40	20	40
24.5	80	20	0

Run time: approximately 25 min

Standard solution:
Accurately weigh between 14.5 and 15.5 mg of the *apo*-12'-lycopenal standard into a 50-ml volumetric flask. Dissolve in toluene stabilised with BHT and make up to volume. Transfer 2 ml of the solution into 100-ml volumetric flask and add toluene stabilised with BHT to volume.

Sample solution:
Accurately weigh between 29.0 and 31.0 mg of the sample into a 10-ml volumetric flask and dissolve and dilute to volume with toluene stabilised with BHT. Put the solution in an ultrasonic bath for 10 min.

Results:
The retention time of *apo*-12'-lycopenal is approximately 14 min. The relative retention time of *apo*-12'-lycopenal with respect to all-*trans*-lycopene is 1.6.

Calculation:

$$Apo\text{-}12'\text{-lycopenal}\,(\%) = \frac{A_s \times W_{St} \times 10}{A_{St} \times W_s \times 2500} \times 100$$

Where:

A_s is the peak area of the sample

A_{St} is the peak area of the standard

W_{St} is the weight of the standard (mg)

W_s is the weight of the sample (mg)

10 is the volume of the volumetric flask in which the sample was dissolved (ml)

2500 is the volume of the volumetric flask in which the standard was dissolved (50 ml) multiplied by dilution (50)

METHOD OF ASSAY Determine total lycopenes and all-*trans*-lycopene by HPLC using the following conditions:

Reagents (Note: all solvents should be HPLC-grade):
Hexane
Tetrahydrofuran stabilised with 0.025% BHT
N-Ethyl-diisopropylamine

Lycopene standard (purity 95% or higher; available from CaroteNature GmbH)

Apparatus:
Spectrophotometer with a 1-cm cuvette
HPLC system with a suitable pump, injector, thermostated column compartment, and integrator

Column:	Two serially-connected two stainless steel columns (250x4.0 mm)
Stationary phase:	Nucleosil 300-5, 5 µm (Macherey-Nagel or equivalent)
Detector:	UV/VIS or VIS

HPLC conditions:

Flow rate:	0.8 ml/min
Injection volume:	20µl
Pressure:	approx. 80 bar
Column temperature:	20°
Detection:	470 nm
Mobile phase:	0.15% solution of N-ethyl-diisopropylamine in hexane (v/v)
Run time:	30 min

HPLC standard solution:
Accurately weigh between 5.5 and 6.5 mg of the lycopene standard into a 100-ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran stabilised with BHT and make up to volume with hexane. This is a standard solution for the HPLC assay.

Spectrophotometric standard solution:
Transfer 5.0 ml of the HPLC standard solution into a 100-ml volumetric flask and make up to volume with hexane. This is a standard solution for the spectrophotometric determination of lycopene in the lycopene standard.

Sample solution:
Accurately weigh between 4.5 and 5.5 mg of the sample into a 100-ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran stabilised with BHT and make up to volume with hexane.

Spectrophotometric determination of lycopene:
Measure the absorbance of the spectrophotometric standard solution in a 1-cm cuvette at the wavelength of maximum absorption (approximately 470 nm). Use hexane as the blank.

Calculation:

$$C_{St}\,(mg/l) = \frac{A \times 10000}{3450}$$

Where:
C_{St} is the lycopene concentration in the spectrophotometric standard solution (mg/l)

A is absorbance at the wavelength of maximum absorption

3450 is the specific absorbance $A^{1\%}_{1cm}$ of all-*trans*-lycopene in hexane

10000 is the scaling factor

HPLC analysis:
Repeatedly inject 20 µl of the HPLC standard solution. Record the total peak area of all detected lycopene isomers (exclude the solvent peak). Calculate the mean peak area from repeated injections and calculate the lycopene response factor (RF) according to the formula:

$$RF = \frac{A_{St}}{C_{St} \times 20}$$

Where:

RF is the response factor of lycopene (AU x l/mg)

A_{St} is the mean peak area of all lycopene peaks (AU)

C_{St} is the concentration of lycopene in the spectrophotometric standard solution (mg/l)

20 is the dilution factor used in the preparation of the spectrophotometric standard solution from the HPLC standard solution.

Inject the sample solution and record the peak areas of lycopene isomers.

Results:
Retention times:

Lycopene isomer	Relative retention time*	Absolute retention time (approx.)
13-*cis*-lycopene	0.6	14 min
9-*cis*-lycopene	0.8	19 min
All-*trans*-lycopene	1.0	22 min
5-*cis*-lycopene	1.1	24 min

* relative to all-*trans*-lycopene

Calculations:

Calculate the content of total lycopenes according to the formula:

$$\text{Total lycopenes (\%)} = \frac{(A_{trans} + A_{5cis} + A_{9cis} + A_{13cis} + A_{xcis}) \times 0.1}{RF \times W_s} \times 100$$

Where:

A_{trans} is the peak area of all-*trans*-lycopene (AU)

A_{5cis}, A_{9cis}, and A_{13cis} are the peak areas of 5*cis*-, 9*cis*-, and 13*cis*-lycopene (AU)

A_{xcis} is the peak area of other *cis* isomers, if detected (AU)

0.1 is the volume of the flask in which the sample was dissolved (l)

RF is the response factor of lycopene (AU x l/mg)

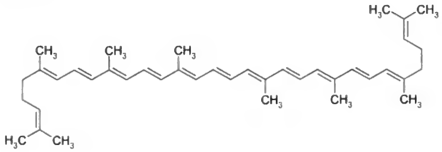
W_s is the weight of the sample (mg)

Calculate the content of all-*trans*-lycopene as follows:

$$\text{All-}\textit{trans}\text{-lycopene}\ (\%) = \frac{A_{trans} \times 0.1}{RF \times W_s} \times 100$$

LYCOPENE FROM *BLAKESLEA TRISPORA*

New specifications prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006). A group ADI of 0-0.5 mg/kg bw for synthetic lycopene and lycopene derived from Blakeslea trispora was established at the 67th JECFA (2006).

SYNONYMS	INS 160d
DEFINITION	Lycopene from *Blakeslea trispora* is extracted from the fungal biomass and purified by crystallization and filtration. It consists predominantly of all-*trans*-lycopene. It also contains minor quantities of other carotenoids. Isopropanol and isobutyl acetate are the only solvents used in the manufacture. Commercial lycopene preparations intended for use in food are formulated either as suspensions in edible oils or as water-dispersible powders and are stabilised with antioxidants.
Chemical names	Ψ,Ψ-carotene all-*trans*-lycopene (all-E)-lycopene (all-E)-2,6,10,14,19,23,27,31-octamethyl-2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene
C.A.S. number	502-65-8
Chemical formula	$C_{40}H_{56}$
Structural formula	
Formula weight	536.9
Assay	Not less than 95% total lycopenes; not less than 90% all-*trans*-lycopene
DESCRIPTION	Red crystalline powder
FUNCTIONAL USES	Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Insoluble in water, freely soluble in chloroform
Test for carotenoids	The colour of the solution of the sample in acetone disappears after

successive additions of a 5% solution of sodium nitrite and 1N sulfuric acid

<u>Solution in chloroform</u> A 1% solution is clear and has intensive red-orange colour

<u>Spectrophotometry</u> (Vol. 4) A solution in hexane shows an absorption maximum at approximately 470 nm

PURITY

<u>Other carotenoids</u> Not more than 5%
See description under METHOD OF ASSAY

<u>Loss on drying</u> (Vol. 4) Not more than 0.5% (40°, 4 h at 20 mm Hg)

<u>Lead</u> (Vol. 4) Not more than 1 mg/kg
Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

<u>Residual solvents</u> (Vol. 4) Isopropanol: Not more than 0.1%
Isobutyl acetate: Not more than 1.0%

METHOD OF ASSAY

The HPLC method of assay is suitable for determination of total lycopenes (all-*trans*-lycopene and *cis*-lycopene isomers), all-*trans*-lycopene, and other carotenoids. (Note: the predominant *cis* isomer detected in lycopene from *B. trispora* is 13-*cis*-lycopene.)

<u>Reagents</u> (Note: all solvents should be HPLC-grade):
Acetonitrile
Methanol
Acetone
Hexane
Methylene chloride
Lycopene standard (purity 95% or higher; available from Vitatene S.A.)

<u>Apparatus</u>:
VIS or UV/VIS spectrophotometer with a 1-cm light path optical cell
HPLC system with either a VIS or UV/VIS detector or a suitable diode array detector, injector, column oven, and integrator
Column: Vydac 218 TP54 5 m (4.6x250 mm) or equivalent

<u>HPLC conditions</u>:

Mobile phase:	acetonitrile/methanol (40:60)
Flow rate:	1 ml/min
Detection:	470 nm
Injection volume:	10 µl
Column temperature:	30°
Injector temperature:	10°
Run time:	15 min

<u>Standard solution</u>:
Weigh accurately about 25 mg lycopene standard into a 100-ml volumetric flask. Dissolve in 10 ml of methylene chloride and add

hexane to volume. Pipet 1 ml of the above solution into a 50-ml volumetric flask and add acetone to volume.

Sample solution:
Prepare as the standard solution.

HPLC analysis:
Chromatograph the standard solution. The retention time of all-*trans*-lycopene is approximately 11.5 to 12.5 min. The relative retention time of 13-*cis*-lycopene with respect to all-*trans*-lycopene is 1.25. The relative retention times for other carotenoids with respect to all-*trans*-lycopene are 1.2 for β-carotene and 1.1 for γ-carotene.

Record the total peak area of all-*trans*-lycopene and *cis*-lycopene isomers and calculate the response factor (RF) for lycopene as follows:

$$RF = \frac{Ast \times 5000}{Wst \times Pst}$$

Where:

RF is the response factor for lycopene (AU ml/mg)

Ast is the total lycopene (all-*trans*-lycopene + *cis*-lycopene isomers) peak area

5000 is the volume of the volumetric flask in which the standard was dissolved (100 ml) multiplied by dilution (50)

Wst is the weight of the standard (mg)

Pst is the purity of the standard expressed as a proportion of lycopene in the lycopene standard (determined as described under Standard purity determination)

Chromatograph the sample solution and record the following peak areas:
A1 – all-*trans* lycopene
A2 – total lycopene (all-*trans*-lycopene + *cis*-lycopene isomers)
A3 – other carotenoids
A4 – all carotenoids (all-*trans*-lycopene + *cis*-lycopene isomers + other carotenoids)

Results:
Calculate the % of total lycopenes, all-*trans*-lycopene, and other carotenoids as follows:

$$\text{Total lycopenes (\%)} = \frac{A2 \times 5000}{W \times RF} \times 100$$

$$\text{All-}\textit{trans}\text{-lycopene (\%)} = \frac{A1}{A2} \times 100$$

$$\text{Other carotenoids (\%)} = \frac{A3}{A4} \times 100$$

Where:

W is the sample weight (mg)

RF is the response factor (AU ml/mg)

5000 is the volume of the volumetric flask in which the standard was dissolved (100 ml) multiplied by dilution (50)

Standard purity determination:

Accurately weigh about 20 mg of the lycopene standard into a 100-ml volumetric flask. Dissolve in 10 ml of methylene chloride and add hexane to volume. Pipet 1 ml of the solution into a 100-ml volumetric flask and add hexane to volume. Measure the absorbance in a 1-cm optical cell at the wavelength of maximum absorption (approximately 470 nm). Use hexane as the blank.

Calculation:

$$Pst = \frac{Amax \times 10000}{345 \times Wst}$$

Where:

Pst is the purity of the lycopene standard calculated as a proportion of lycopene in the lycopene standard (Note: Pst equals 1 for a 100% pure standard and is less than 1 for a standard with purity below 100%)

Amax is the absorbance at the wavelength of maximum absorption

Wst is the weight of the standard (mg)

10000 is the volume of the volumetric flask in which lycopene was dissolved (100 ml) multiplied by dilution (100)

345 is the absorptivity of lycopene in hexane

DL-MALIC ACID

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 57th JECFA (1999) and published in FNP 52 Add 9 (2001) and the Combined Compendium of Food Additive specifications FAO JECFA Monographs 1 (2005). An ADI "not specified" was established at the 13th JECFA (1969).

SYNONYMS 2-Hydroxybutanedioic acid; INS No. 296

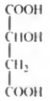
DEFINITION

Chemical names DL-Malic acid, 2-Hydroxybutanedioic acid, Hydroxysuccinic acid

C.A.S. number 6915-15-7

Chemical formula $C_4H_6O_5$

Structural formula



Formula weight 134.1

Assay Not less than 99.0%

DESCRIPTION White or nearly white crystalline powder or granules

FUNCTIONAL USES Acidity regulator

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Very soluble in water; freely soluble in ethanol

Melting range (Vol. 4) 127 - 132°

Test for malate (Vol. 4) Passes test
Test 5 ml of a 1 in 20 solution of the sample, neutralized with ammonia TS

PURITY

Fumaric and maleic acid (Vol. 4)	Not more than 1.0% of fumaric acid and not more than 0.05% of maleic acid
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.
METHOD OF ASSAY	Dissolve about 2 g of the sample, accurately weighed, in 40 ml of recently boiled and cooled water, add 2 drops of phenolphthalein TS and titrate with 1 N sodium hydroxide to the first appearance of a faint pink colour which persists for at least 30 sec. Each ml of 1 N sodium hydroxide is equivalent to 67.04 mg of $C_4H_6O_5$.

SODIUM HYDROGEN DL-MALATE

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 26th JECFA (1982) and published in FNP 52 (1992) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). Heavy metals and arsenic specifications were revised at the 59th JECFA (2002). An ADI 'not specified' was established at the 26th JECFA (1982).

SYNONYMS Malic acid monosodium salt; INS No. 350(i)

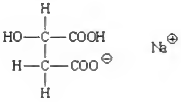
DEFINITION

Chemical names: Monosodium DL-malate, 2-hydroxybutanedioic acid monosodium salt

C.A.S. number: 58214-38-3

Chemical formula: $C_4H_5NaO_5$

Structural formula:



Formula weight: 156.1

Assay: Not less than 99.0% on the dried basis

DESCRIPTION Odourless white powder

FUNCTIONAL USES Buffering agent, humectant

CHARACTERISTICS

IDENTIFICATION

Test for sodium (Vol. 4): Passes test

Test for malate (Vol. 4): Passes test
Test 5 ml of a 1 in 20 solution of the sample

PURITY

Loss on drying (Vol. 4): Not more than 2% (110°, 3 h)

Fumaric and maleic acid (Vol. 4)	Not more than 1.0% of fumaric acid and not more than 0.05% of maleic acid
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

METHOD OF ASSAY

Weigh accurately about 1.5 g of the dried sample and transfer into a platinum or porcelain crucible of 20 to 30 mm in diameter. Heat very gently, and gradually raise the temperature. Continue heating for 2 h, and carbonize thoroughly. The heating temperature is between 300° and 400°, at which the crucible shows a dull red colour. If a gas burner is used, the flame should not contact with the carbonized mass. After allowing the carbonized mass to cool, disintegrate with a glass rod, and transfer the mass and crucible into a beaker. Add 50 ml of water and 50 ml of 0.5 N sulfuric acid, cover the beaker with a watch glass, heat the contents on a water bath for 1 h, and filter. If the filter is coloured, weigh the sample again, and carbonize it thoroughly. Wash the beaker, the crucible and the residue on the filter paper with hot water until the washings become neutral to blue litmus paper. Combine the washings to the filtrate. Titrate an excess of sulfuric acid with 0.5 N sodium hydroxide, using 3 drops of methyl red TS as the indicator. Each ml of 0.5 N sulfuric acid is equivalent to 78.04 mg of $C_4H_5NaO_5$.

SODIUM DL-MALATE

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 30th JECFA (1986) and published in FNP 52 (1992) and in the Combined Compendium of Food Additive specifications, FAO JECFA monographs 1 (2005). Metals and arsenic specifications were revised at the 59th JECFA (2002). An ADI 'not specified' was established at the 23rd JECFA (1979).

SYNONYMS Malic acid sodium salt; INS No. 350(ii)

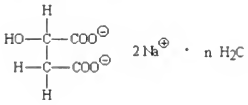
DEFINITION

Chemical names: Disodium DL-malate, hydroxybutanedioic acid disodium salt

C.A.S. number: 676-46-0

Chemical formula: Hemihydrate: $C_4H_4Na_2O_5 \cdot 1/2\ H_2O$
Trihydrate: $C_4H_4Na_2O_5 \cdot 3\ H_2O$

Structural formula



Formula weight: Hemihydrate: 187.1
Trihydrate: 232.1

Assay: Not less than 98% and not more than 102% on the dried basis

DESCRIPTION Odourless white crystalline powder or lumps

FUNCTIONAL USES Acidity regulator

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4): Freely soluble in water

Test for sodium (Vol. 4): Passes test

Test for malate (Vol. 4): Passes test
Test 5 ml of a 1 in 20 solution of the sample

PURITY

Loss on drying (Vol. 4)	Hemihydrate: Not more than 7% (130°, 4 h) Trihydrate: 20.5% - 23.5% (130°, 4 h)
Alkalinity	Not more than 0.2% as Na_2CO_3 Dissolve 1 g of the sample in 20 ml of freshly boiled and cooled water, and add 2 drops of phenolphthalein TS. If a pink colour is produced, add 0.4 ml of 0.1 N sulfuric acid. The colour of the solution disappears.
Fumaric and maleic acid (Vol. 4)	Not more than 1.0% of fumaric acid and not more than 0.05% of maleic acid
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

METHOD OF ASSAY Dissolve about 0.25 g of the dried sample, accurately weighed, in 50 ml of glacial acetic acid, and titrate with 0.1 N perchloric acid, determining the endpoint potentiometrically. Each ml of 0.1 N perchloric acid is equivalent to 8.903 mg of $C_4H_4Na_2O_5$.

TITANIUM DIOXIDE

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 63rd JECFA (2004) and published in FNP 52 Add 12 (2004) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). An ADI "not limited" was established at the 13th JECFA (1969).

SYNONYMS Titania, CI Pigment white 6, CI (1975) No. 77891, INS No. 171

DEFINITION Titanium dioxide is produced by either the sulfate or the chloride process. Processing conditions determine the form (anatase or rutile structure) of the final product.

In the sulfate process, sulfuric acid is used to digest ilmenite ($FeTiO_3$) or ilmenite and titanium slag. After a series of purification steps, the isolated titanium dioxide is finally washed with water, calcined, and micronized.

In the chloride process, chlorine gas is reacted with a titanium-containing mineral under reducing conditions to form anhydrous titanium tetrachloride, which is subsequently purified and converted to titanium dioxide either by direct thermal oxidation or by reaction with steam in the vapour phase. Alternatively, concentrated hydrochloric acid can be reacted with the titanium-containing mineral to form a solution of titanium tetrachloride, which is then further purified and converted to titanium dioxide by hydrolysis. The titanium dioxide is filtered, washed, and calcined.

Commercial titanium dioxide may be coated with small amounts of alumina and/or silica to improve the technological properties of the product.

C.A.S. number 13463-67-7

Chemical formula TiO_2

Formula weight 79.88

Assay Not less than 99.0% on the dried basis (on an aluminium oxide and silicon dioxide-free basis)

DESCRIPTION White to slightly coloured powder

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) — Insoluble in water, hydrochloric acid, dilute sulfuric acid, and organic solvents. Dissolves slowly in hydrofluoric acid and hot concentrated sulfuric acid.

Colour reaction — Add 5 ml sulfuric acid to 0.5 g of the sample, heat gently until fumes of sulfuric acid appear, then cool. Cautiously dilute to about 100 ml with water and filter. To 5 ml of this clear filtrate, add a few drops of hydrogen peroxide; an orange-red colour appears immediately.

PURITY

Loss on drying (Vol. 4) — Not more than 0.5% (105°, 3 h)

Loss on ignition (Vol. 4) — Not more than 1.0% (800°) on the dried basis

Aluminium oxide and/or silicon dioxide — Not more than 2%, either singly or combined
See descriptions under TESTS

Acid-soluble substances — Not more than 0.5%; Not more than 1.5% for products containing alumina or silica.
Suspend 5 g of the sample in 100 ml 0.5 N hydrochloric acid and place on a steam bath for 30 min with occasional stirring. Filter through a Gooch crucible fitted with a glass fibre filter paper. Wash with three 10-ml portions of 0.5 N hydrochloric acid, evaporate the combined filtrate and washings to dryness, and ignite at a dull red heat to constant weight.

Water-soluble matter (Vol. 4) — Not more than 0.5%
Proceed as directed under acid-soluble substances (above), using water in place of 0.5 N hydrochloric acid.

Impurities soluble in 0.5 N hydrochloric acid

Antimony — Not more than 2 mg/kg
See description under TESTS

Arsenic — Not more than 1 mg/kg
See description under TESTS

Cadmium — Not more than 1 mg/kg
See description under TESTS

Lead — Not more than 10 mg/kg
See description under TESTS

Mercury (Vol. 4) — Not more than 1 mg/kg
Determine using the cold vapour atomic absorption technique. Select a sample size appropriate to the specified level

TESTS

PURITY TESTS

Impurities soluble in 0.5 N hydrochloric acid

Antimony, arsenic, cadmium and lead (Vol.4)

Transfer 10.0 g of sample into a 250-ml beaker, add 50 ml of 0.5 *N* hydrochloric acid, cover with a watch glass, and heat to boiling on a hot plate. Boil gently for 15 min, pour the slurry into a 100- to 150-ml centrifuge bottle, and centrifuge for 10 to 15 min, or until undissolved material settles. Decant the supernatant extract through a Whatman No. 4 filter paper, or equivalent, collecting the filtrate in a 100-ml volumetric flask and retaining as much as possible of the undissolved material in the centrifuge bottle. Add 10 ml of hot water to the original beaker, washing off the watch glass with the water, and pour the contents into the centrifuge bottle. Form a slurry, using a glass stirring rod, and centrifuge. Decant through the same filter paper, and collect the washings in the volumetric flask containing the initial extract. Repeat the entire washing process two more times. Finally, wash the filter paper with 10 to 15 ml of hot water. Cool the contents of the flask to room temperature, dilute to volume with water, and mix.

Determine antimony, cadmium, and lead using an AAS/ICP-AES technique appropriate to the specified level. Determine arsenic using the ICP-AES/AAS-hydride technique. Alternatively, determine arsenic using Method II of the Arsenic Limit Test, taking 3 g of the sample rather than 1 g. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

Aluminium oxide

Reagents and sample solutions

0.01 N Zinc Sulfate

Dissolve 2.9 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$) in sufficient water to make 1000 ml. Standardize the solution as follows: Dissolve 500 mg of high-purity (99.9%) aluminium wire, accurately weighed, in 20 ml of concentrated hydrochloric acid, heating gently to effect solution, then transfer the solution into a 1000-ml volumetric flask, dilute to volume with water, and mix. Transfer a 10 ml aliquot of this solution into a 500 ml Erlenmeyer flask containing 90 ml of water and 3 ml of concentrated hydrochloric acid, add 1 drop of methyl orange TS and 25 ml of 0.02 M disodium ethylenediaminetetraacetate (EDTA) Add, dropwise, ammonia solution (1 in 5) until the colour is just completely changed from red to orange-yellow. Then, add:

(a): 10 ml of ammonium acetate buffer solution (77 g of ammonium acetate plus 10 ml of glacial acetic acid, dilute to 1000 ml with water) and

(b): 10 ml of diammonium hydrogen phosphate solution (150 g of diammonium hydrogen phosphate in 700 ml of water, adjusted to pH 5.5 with a 1 in 2 solution of hydrochloric acid, then dilute to 1000 ml with water).

Boil the solution for 5 min, cool it quickly to room temperature in a stream of running water, add 3 drops of xylenol orange TS, and mix.

Using the zinc sulfate solution as titrant, titrate the solution to the first yellow-brown or pink end-point colour that persists for 5-10 sec. (*Note:* This titration should be performed quickly near the end-point by adding rapidly 0.2 ml increments of the titrant until the first colour change occurs; although the colour will fade in 5-10 sec, it is the true

end-point. Failure to observe the first colour change will result in an incorrect titration. The fading end-point does not occur at the second end-point.)

Add 2 g of sodium fluoride, boil the mixture for 2-5 min, and cool in a stream of running water. Titrate this solution, using the zinc sulfate solution as titrant, to the same fugitive yellow-brown or pink end-point as described above.

Calculate the titre T of zinc sulfate solution by the formula:

$$T = 18.896\ W / V$$

where

T is the mass (mg) of Al_2O_3 per ml of zinc sulfate solution
W is the mass (g) of aluminium wire
V is the ml of the zinc sulfate solution consumed in the second titration
18.896 = (R × 1000 mg/g × 10 ml/2)/1000 ml and
R is the ratio of the formula weight of aluminium oxide to that of elemental aluminium.

Sample Solution A
Accurately weigh 1 g of the sample and transfer to a 250-ml high-silica glass Erlenmeyer flask. Add 10 g of sodium bisulfate ($NaHSO_4 \cdot H_2O$). (*Note*: Do not use more sodium bisulfate than specified, as an excess concentration of salt will interfere with the EDTA titration later on in the procedure.) Begin heating the flask at low heat on a hot plate, and then gradually raise the temperature until full heat is reached. (*Caution:* perform this procedure in a well ventilated area.) When spattering has stopped and light fumes of SO_3 appear, heat in the full flame of a Meeker burner, with the flask tilted so that the fusion of the sample and sodium bisulfate is concentrated at one end of the flask. Swirl constantly until the melt is clear (except for silica content), but guard against prolonged heating to avoid precipitation of titanium dioxide. Cool, add 25 ml sulfuric acid solution (1 in 2), and heat until the mass has dissolved and a clear solution results. Cool, and dilute to 120 ml with water. Introduce a magnetic stir bar into the flask.

Sample Solution B
Prepare 200 ml of an approximately 6.25 M solution of sodium hydroxide. Add 65 ml of this solution to Sample Solution A, while stirring with the magnetic stirrer; pour the remaining 135 ml of the alkali solution into a 500-ml volumetric flask.

Slowly, with constant stirring, add the sample mixture to the alkali solution in the 500-ml volumetric flask; dilute to volume with water, and mix. (*Note*: If the procedure is delayed at this point for more than 2 hours, store the contents of the volumetric flask in a polyethylene bottle.) Allow most of the precipitate to settle (or centrifuge for 5 min), then filter the supernatant liquid through a very fine filter paper. Label the filtrate Sample Solution B.

Sample Solution C
Transfer 100 ml of the Sample Solution B into a 500-ml Erlenmeyer flask, add 1 drop of methyl orange TS, acidify with hydrochloric acid solution (1 in 2), and then add about 3 ml in excess. Add 25 ml of 0.02

M disodium EDTA, and mix. [*Note*: If the approximate Al_2O_3 content is known, calculate the optimum volume of EDTA solution to be added by the formula: (4 x % Al_2O_3) + 5.]

Add, dropwise, ammonia solution (1 in 5) until the colour is just completely changed from red to orange-yellow. Then add10 ml each of *Solutions 1* and *2* (see above) and boil for 5 min. Cool quickly to room temperature in a stream of running water, add 3 drops of xylenol orange TS, and mix. If the solution is purple, yellow-brown, or pink, bring the pH to 5.3 - 5.7 by the addition of acetic acid. At the desired pH, a pink colour indicates that not enough of the EDTA solution has been added, in which case, discard the solution and repeat this procedure with another 100 ml of Sample Solution B, using 50 ml, rather than 25 ml, of 0.02 M disodium EDTA.

Procedure

Using the standardized zinc sulfate solution as titrant, titrate Sample Solution C to the first yellow-brown or pink end-point that persists for 5-10 sec. (*Important:* See Note under "*0.01 Zinc sulfate*".) This first titration should require more than 8 ml of titrant, but for more accurate work a titration of 10-15 ml is desirable.

Add 2 g of sodium fluoride to the titration flask, boil the mixture for 2-5 min, and cool in a stream of running water. Titrate this solution, using the standardized zinc sulfate solution as titrant, to the same fugitive yellow-brown or pink end-point as described above.

Calculation:

Calculate the percentage of aluminium oxide (Al_2O_3) in the sample taken by the formula:

$$\% \ Al_2O_3 = 100 \times (0.005VT)/S$$

where

V is the number of ml of 0.01 N zinc sulfate consumed in the second titration,
T is the titre of the zinc sulfate solution,
S is the mass (g) of the sample taken, and
0.005 = 500 ml / (1000mg/g × 100 ml).

Silicon dioxide

Accurately weigh 1 g of the sample and transfer to a 250-ml high-silica glass Erlenmeyer flask. Add 10 g of sodium bisulfate ($NaHSO_4 \cdot H_2O$). Heat gently over a Meeker burner, while swirling the flask, until decomposition and fusion are complete and the melt is clear, except for the silica content, and then cool. (*Caution:* Do not overheat the contents of the flask at the beginning, and heat cautiously during fusion to avoid spattering.)

To the cooled melt add 25 ml of sulfuric acid solution (1 in 2) and heat carefully and slowly until the melt is dissolved. Cool, and carefully add 150 ml of water by pouring very small portions down the sides of the flask, with frequent swirling to avoid over-heating and spattering. Allow the contents of the flask to cool, and filter through fine ashless filter paper, using a 60 degree gravity funnel. Rinse out all the silica from the flask onto the filter paper with sulfuric acid solution (1 in 10). Transfer the filter paper and its contents into a platinum crucible, dry in an oven at 120^0, and heat the partly covered crucible over a Bunsen

burner. To prevent flaming of the filter paper, first heat the cover from above, and then the crucible from below.

When the filter paper is consumed, transfer the crucible to a muffle furnace and ignite at 1000° for 30 min. Cool in a desiccator, and weigh. Add 2 drops of sulfuric acid (1 in 2) and 5 ml of concentrated hydrofluoric acid (sp.gr. 1.15), and carefully evaporate to dryness, first on a low-heat hot plate (to remove the HF) and then over a Bunsen burner (to remove the H_2SO_4). Take precautions to avoid spattering, especially after removal of the HF. Ignite at 1000° for 10 min, cool in a desiccator, and weigh again. Record the difference between the two weights as the content of SiO_2 in the sample.

METHOD OF ASSAY Accurately weigh about 150 mg of the sample, previously dried at 105° for 3 hours, and transfer into a 500-ml conical flask. Add 5 ml of water and shake until a homogeneous, milky suspension is obtained. Add 30 ml of sulfuric acid and 12 g of ammonium sulfate, and mix. Initially heat gently, then heat strongly until a clear solution is obtained. Cool, then cautiously dilute with 120 ml of water and 40 ml of hydrochloric acid, and stir. Add 3 g of aluminium metal, and immediately insert a rubber stopper fitted with a U-shaped glass tube while immersing the other end of the U-tube into a saturated solution of sodium bicarbonate contained in a 500-ml wide-mouth bottle, and generate hydrogen. Allow to stand for a few minutes after the aluminium metal has dissolved completely to produce a transparent purple solution. Cool to below 50° in running water, and remove the rubber stopper carrying the U-tube. Add 3 ml of a saturated potassium thiocyanate solution as an indicator, and immediately titrate with 0.2 N ferric ammonium sulfate until a faint brown colour that persists for 30 seconds is obtained. Perform a blank determination and make any necessary correction. Each ml of 0.2 N ferric ammonium sulfate is equivalent to 7.990 mg of TiO_2.

ZEAXANTHIN (SYNTHETIC)

Prepared at the 67th JECFA (2006) and published in FAO JECFA Monographs 3 (2006), superseding specifications prepared at the 63rd JECFA (2004) and published in FNP 52 Add 12 (2004) and in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). A group ADI of 0 – 2 mg/kg bw for lutein and zeaxanthin (synthetic) was established at the 63rd JECFA (2004).

SYNONYMS INS No. 161h(i)

DEFINITION The synthetic all-trans isomer of zeaxanthin is produced by the Wittig condensation from synthetic intermediates commonly used in the production of other carotenoids used in foods.

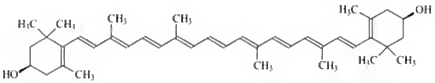
Chemical Names: (all-E)-1,1'-(3,7,12,16-Tetramethyl-1,3,5,7,9,11,13,15,17-octadecanonaene-1,18-diyl)bis[2,6,6-trimethylcyclohexene-3-ol]

3R,3'R-β, β -Carotene-3,3'-diol

C.A.S. number: 144-68-3

Chemical formula: $C_{40}H_{56}O_2$

Structural formula



Formula weight: 568.9

Assay: Not less than 96%

DESCRIPTION Orange-red crystalline powder, with little or no odour

FUNCTIONAL USES Colour, nutrient supplement

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4): Sparingly soluble in chloroform, practically insoluble in water and ethanol

Test for carotenoid: The colour of the solution of the sample in acetone disappears after successive additions of a 5 % solution of sodium nitrite and 1N sulfuric acid

Spectrophotometry (Vol. 4): An ethanol solution of the sample shows maximum absorption between 450 and 454 nm

PURITY

Loss on drying (Vol. 4)	Not more than 0.2 % (80° under reduced pressure for 18 h in the presence of P_2O_5)
cis-Zeaxanthins	Not more than 2.0 % See description under METHOD OF ASSAY
12'-Apo-zeaxanthinal, diatoxanthin, parasilo-xanthin	Not more than 1.1 % combined See description under METHOD OF ASSAY
Triphenyl phosphine oxide (TPPO) (Vol. 4)	Not more than 0.01%
Lead (Vol. 4)	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4.

METHOD OF ASSAY

The HPLC method of assay is designed to determine *trans*-zeaxanthin, the *cis*-isomers of zeaxanthins and zeaxanthin-related impurities: 12'-apo-zeaxanthinal, parasiloxanthin, and diatoxanthin. (NOTE: All solvents should be HPLC grade.)

Standards
Trans-zeaxanthin, 12´-apo-zeaxanthinal, and diatoxanthin.
(All *trans*-zeaxanthin, 12´-apo-zeaxanthinal, and diatoxanthin available from DSM Nutritional Products, Kaiseraugst, Switzerland. All-*trans*-zeaxanthin is also available from Fluka, Buchs, Switzerland).

Standard solutions:
Solution 1: Accurately weigh 34 to 36 mg of 12'-apo-zeaxanthinal and transfer to a 100-ml volumetric flask. Add tetrahydrofuran to dissolve the substance and bring to volume.

Solution 2: Accurately weigh 34 to 36 mg of diatoxanthin and transfer to a 100-ml volumetric flask. Add tetrahydrofuran to dissolve the substance and bring to volume.

Working standard: Accurately weigh 69.0 to 71.0 mg of *trans*-zeaxanthin and transfer to a 100-ml volumetric flask. Add 50 ml of tetrahydrofuran, 1 ml of standard solution 1, and 1 ml of standard solution 2. Bring to volume with tetrahydrofuran.

Sample solution:
Accurately weigh 69.0 to 71.0 mg of sample and dissolve in 100 ml of tetrahydrofuran.

Mobile phase:
In a 2000-ml volumetric flask containing a small quantity of hexane, add 400 ml of ethyl acetate, 20 ml of 2-methoxyethanol, and 2.0 ml of *N*-ethyldiisopropylamine. Bring to volume with hexane.

Chromatography apparatus and conditions:

Column:	Stainless steel; 250 x 4 mm
Column temperature:	25°
Stationary phase:	Spherisorb Si, 3 µm or similar
Flow:	Flow 1.0 ml/min
Detector:	VIS 450 nm
Injection:	2.0 µl
Run time:	35 min

Procedure:
Inject a 2.0 µl aliquot of the Working standard and measure the area of the peaks for *trans*-zeaxanthin, 12´-apo-zeaxanthinal, and diatoxanthin. Inject a 2.0 µl aliquot of the sample solution and measure the areas of the peaks for *trans*-zeaxanthin, *cis*-isomers of zeaxanthins, 12´-apo-zeaxanthinal, parasiloxanthin, and diatoxanthin. Typical retention times and relative retention times are shown in the table below.

Substance	Relative retention time*	Approx. absolute retention time [min]
trans-zeaxanthin	1.00	17.7
cis isomers of zeaxanthin	1.38 – 1.46	24.4 – 25.8
12´-apo-zeaxanthinal	0.46	8.2
parasiloxanthin	0.96	17.0
diatoxanthin	1.16	20.5

* in relation to trans-zeaxanthin

Calculation:
Calculate the percentage content of *trans*-zeaxanthin in the sample using the equation below:

$$(\%) = \frac{A_{(s)} \cdot W_{(R)} \cdot P_{(R)} \cdot 100}{A_{(R)} \cdot W_{(s)}}$$

Where:

- $A_{(S)}$ is the peak area of substance to be determined in the sample solution
- $W_{(R)}$ is the weight (mg) of substance in the Working standard
- $P_{(R)}$ is the purity of substance (0.98 if purity is 98%) in the Working standard
- $A_{(R)}$ is the peak area of substance in the Working standard
- $W_{(S)}$ is the weight (mg) of the sample in the sample solution

To calculate the percentage of the contents of 12´-apo-zeaxanthinal, and diatoxanthin use the equation above and the corresponding weights and purity for each substance.

Cis-isomers of zeaxanthin and parasiloxanthin are not included in the standard solutions. However, their absorptivities at the wavelength employed in the method are the same as the absorptivity for *trans*-zeaxanthin. Their percentage contents can therefore be calculated using the above formula considering their weights to be the same as that of *trans*-zeaxanthin and their purities ($P_{(R)}$) to be equal to one (100% purity = 1).

WITHDRAWAL OF SPECIFICATIONS FOR CERTAIN FOOD ADDITIVES

Butyl para-hydroxybenzoate (butyl paraben) and propyl para-hydroxybenzoate (propylparaben)

The JECFA specifications monographs for butyl para-hydroxybenzoate and propyl para-hydroxybenzoate were withdrawn as butyl-paraben and propyl-paraben were excluded from the group ADI of the parabens by the Committee at the 67^{th} meeting.

Ethylene oxide

The JECFA specifications monograph for ethylene oxide was withdrawn by the Committee at the 67^{th} meeting, in view of the fact that ethylene oxide has never been used as a food additive as such and the known hazards of ethylene oxide.

GENERAL SPECIFICATIONS AND CONSIDERATIONS FOR ENZYMES USED IN FOOD PROCESSING

The following general specifications were prepared by the Committee at its sixty-seventh meeting (2006) for publication in FAO JECFA Monographs 3 (2006), superseding the general specifications prepared at the fifty-seventh meeting (*1*) and published in FAO JECFA Monographs 1 (*2*). These specifications were originally prepared by the Committee at its twenty-fifth meeting (*3*) and published in FAO Food and Nutrition Papers No. 19 and No. 31/2 (*4,5*). Subsequent revisions were made by the Committee at its thirty-fifth meeting and published in FAO Food and Nutrition Paper No. 52 (*6*). Additional amendments were made at the fifty-first meeting and published in FAO Food and Nutrition Paper No. 52 Add. 6 (*7*), and at the fifty-third meeting (*8*) and partially published in FAO Food and Nutrition Paper No. 52 Add. 7 (9).

Classification and nomenclature of enzymes

Enzymes are proteins that catalyse chemical reactions. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (formerly the International Union of Biochemistry) classified enzymes into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (*10*). Based on the type of reaction catalysed, enzymes are assigned to one of these classes and given an Enzyme Commission (EC) number, a systematic name, and a common name. Other names are also provided, if available. Enzymes used in food processing are often referred to by their common or traditional names such as protease, amylase, malt, or rennet. For enzymes derived from microorganisms, the name of the source microorganism is usually specified, for example, "α-amylase from *Bacillus subtilis*." For enzymes derived from microorganisms modified by using recombinant DNA techniques (referred to as recombinant-DNA microorganisms or genetically modified microorganisms), the names of both the enzyme source (donor organism) and the production microorganism are provided, for example, "α-amylase from *Bacillus licheniformis* expressed in *Bacillus subtilis*."

Enzyme preparations

Enzymes are used in food processing as enzyme preparations. An enzyme preparation contains an active enzyme (in some instances a blend of two or more enzymes) and intentionally added formulation ingredients such as diluents, stabilizing agents, and preserving agents. The formulation ingredients may include water, salt, sucrose, sorbitol, dextrin, cellulose, or other suitable compounds. Enzyme preparations may also contain constituents of the source organism (i.e. an animal, plant, or microbial material from which an enzyme was isolated) and compounds derived from the manufacturing process, for example, the residues of the fermentation broth. Depending on the application, an enzyme preparation may be formulated as a liquid, semi-liquid or dried product. The colour of an enzyme preparation may vary from colourless to dark brown. Some enzymes are immobilized on solid support materials.

Active components

Enzyme preparations usually contain one principal enzyme that catalyses one specific reaction during food processing. For example, α-amylase catalyses the hydrolysis of 1,4-α-D-glucosidic linkages in starch and related polysaccharides. However, some enzyme preparations contain a mixture of enzymes that catalyse two or more different reactions in food. Each principal enzyme present in an enzyme preparation is characterized by its systematic name, common name, and EC number. The activity of each enzyme is measured using an appropriate assay and expressed in defined activity units per weight (or volume) of the preparation.

Source materials

Enzymes used in food processing are derived from animal, plant, and microbial sources. Animal tissues used for the preparation of enzymes should comply with meat inspection requirements and be handled in accordance with good hygienic practice.

Plant material and microorganisms used in the production of enzyme preparations should not leave any residues harmful to health in the processed finished food under normal conditions of use.

Microbial strains used in the production of enzyme preparations may be native strains or mutant strains derived from native strains by the processes of serial culture and selection or mutagenesis and selection or by the application of recombinant DNA technology. Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis (*11–15*). Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Microbial production strains should be taxonomically and genetically characterized and identified by a strain number or other designation. The strain identity may be included in individual specifications, if appropriate. The strains should be maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, should be subjected to methods and culture conditions that are applied consistently and reproducibly from batch to batch. Such conditions should prevent the introduction of microorganisms that could be the source of toxic and other undesirable substances. Culture media used for the growth of microbial sources should consist of components that leave no residues harmful to health in the processed finished food under normal conditions of use.

Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.

Substances used in processing and formulation

Substances used in processing and formulation of enzyme preparations should be suitable for their intended uses.

In the case of immobilized enzyme preparations, leakage of active enzymes, support materials, crosslinking agents and/or other substances used in immobilization should be kept within acceptable limits established in the individual specifications.

To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

$$\% \text{ TOS} = 100 - (A + W + D)$$

where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

Purity

Lead:
Not more than 5 mg/kg
Determine using an atomic absorption spectroscopy/inductively coupled atomic-emission spectroscopy (AAS/ICP-AES) technique appropriate to the specified level. The selection of the sample size and the method of sample preparation may be based on the principles described in the *Compendium of Food Additive Specifications*, Volume 4.

Microbiological criteria:
Salmonella species: absent in 25 g of sample
Total coliforms: not more than 30 per gram
Escherichia coli: absent in 25 g of sample
Determine using procedures described in Volume 4.

Antimicrobial activity:
Absent in preparations from microbial sources.

Other considerations

Safety assessment of food enzyme preparations has been addressed in a number of publications and documents. Pariza & Foster (*11*) proposed a decision tree for determining the safety of microbial enzyme

preparations. Pariza & Johnson (*16*) subsequently updated this decision tree and included information on enzyme preparations derived from recombinant-DNA microorganisms. The Scientific Committee on Food (*17*) issued guidelines for the presentation of data on food enzymes. The document includes a discussion on enzymes from genetically modified organisms including microorganisms, plants, and animals. Several international organizations, government agencies, and expert groups have also published discussion papers or guidelines that address safety assessment of food and food ingredients derived from recombinant-DNA plants and microorganisms (*18–28*). Certain information in these documents may be applicable to enzyme preparations derived from recombinant sources.

An overall safety assessment of each enzyme preparation intended for use in food processing should be performed. This assessment should include an evaluation of the safety of the production organism, the enzyme component, side activities, the manufacturing process, and the consideration of dietary exposure. Evaluation of the enzyme component should include considerations of its potential to cause an allergic reaction. For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:

1. The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.
2. Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance.

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ANALYTICAL METHODS

The following analytical methods were prepared by the Committee at the 67th meeting, for inclusion in the publication of volume 4 of the Combined Compendium of Food Additive Specifications.

Norbixin – determination by HPLC – is referred to in the specifications monographs for the Annatto extracts.

Triphenylphosphine oxide – determination by HPLC – is referred to in the specifications monographs for the Annatto extracts and in the specifications monograph for zeaxanthin (synthetic).

Staphylococcus aureus – direct plate count method for isolation and identification. This method was prepared to allow testing of the limit for *Staphylococcus aureus* spp. included in the specifications monograph for lysozyme hydrochloride. The method for has been edited after the meeting to include additional information pertinent to detection and identification of *Staphylococcus aureus*.

NORBIXIN

Determine by HPLC using the following:

Reagents

Dimethylformamide
Acetonitrile
0.1 M NaOH
Methanol
Acetic acid
Norbixin (purity 99 % or higher; prepare according to the procedure in Scotter *et al.* (1994, 1998) as it is not currently available commercially)

Note: *all solvents should be HPLC-grade*

Apparatus:

HPLC system with a suitable pump, injector, and integrator

Column:	Stainless steel; 250 x 4.6 mm
Stationary phase:	Mixed C8/C18 bonded phase, 5 µm or similar
Detector:	UV/visible

HPLC conditions:

Column temperature:	35°
Mobile phase:	Isocratic 65 % Solution A; 35 % Solution B Solution A: acetonitrile; Solution B: 2 % acetic acid (v/v)
Flow rate:	1.0 ml/min
Injection:	10 µl
Detection:	460 nm
Run time:	40 min

Note: *The retention time of norbixin is approximately 10 min*

Procedure:

Standard solution: Weigh accurately about 25 - 50 mg of the norbixin standard and dissolve in 5 ml of 0.1 M NaOH solution. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with methanol.

Sample solution:
Oil-soluble samples: Weigh accurately about 25 - 50 mg of the sample and dissolve in 3 to 5 ml of dimethylformamide. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with acetonitrile.

Water-soluble samples: Weigh accurately about 25 - 50 mg of the sample and dissolve in 5 ml of 0.1 M NaOH solution. Transfer quantitatively to a 50 ml volumetric flask and dilute to volume with methanol.

Calculation:

$$\text{Norbixin}\,(\%) = \frac{A_s \times W_{St} \times P_{St}}{A_{St} \times W_s} \times 100$$

Where:

As is the peak area of the sample solution
ASt is the peak area of the standard solution
PSt is the purity of the standard expressed as a proportion of Norbixin in the norbixin standard (for example, 0.99 if the standard is 99% pure)
WSt is the weight of the standard (mg)
Ws is the weight of the sample (mg)

References:
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TRIPHENYLPHOSPHINE OXIDE

Determine by HPLC using the following:

Reagents
Hexane
Isopropanol
Tetrahydrofuran (THF)
Triphenylphosphine oxide (TPPO) (purity 99% or higher; ACROS 14043-0250 or equivalent)

Note: *all solvents should be HPLC-grade*

Apparatus:

HPLC system with a suitable pump, injector, and integrator

Column:	Stainless steel; 150 x 4.6 mm
Stationary phase:	Supelcosil LC-Si, 5 µm or similar
Detector:	UV

HPLC conditions:

Column temperature:	20°
Mobile phase:	Isopropanol:hexane (1:24 v/v)
Flow rate:	1.5 ml/min
Injection:	50 µl
Detection:	210 nm
Run time:	10 min

Note: *The retention time of TPPO is approximately 8.1 min*

Procedure:

Standard solution: Weigh accurately about 10 mg of the TPPO standard and dissolve in THF. Transfer quantitatively to a 1000-ml volumetric flask and dilute to volume with THF.

Sample solution: Accurately weigh about 1000 mg of the sample and dissolve in THF. Transfer quantitatively to a 100-ml volumetric flask and dilute to volume with THF.

Calculation:

$$\text{TPPO}\,(\%) = \frac{A_s \times W_{St} \times P_{St} \times 100}{A_{St} \times W_s \times 1000} \times 100$$

Where:
A_s is the peak area of the sample solution
A_{St} is the peak area of the standard solution
P_{St} is the purity of the standard expressed as a proportion of TPPO in the TPPO standard (for example, 0.99 if the standard is 99% pure)
W_{St} is the weight of the standard (mg)
W_s is the weight of the sample (mg)

STAPHYLOCOCCUS AUREUS – Direct plate count method

Note: *This method is suitable for the analysis in which more than 100 S. aureus cells/g may be expected. If the analyst suspects that the number of S. aureus cells is below this limit, then the MPN method should be used. If unknown, both procedures can be used.*

Equipment and materials

1. Drying cabinet, laminar air flow or a well–ventilated room that is free of dust and draft with microbial density of the air in working area not exceeding 15 colonies per plate during a 15-minute exposure.
2. Petri dishes , plastic (15 x 90 mm) or glass (15 x 100 mm)
3. Pipets, 1, 5, and 10 ml, graduated in 0.1 ml units
4. Incubator, 35 $\pm$ 1°
5. Sterile bent glass streaking rods or hockey stick, 3-4 mm diameter, 15-20 cm long with an angled spreading surface 45-55 mm long
6. Colony counter, dark-field, Quebec or equivalent, with suitable light source and grid plate.
7. Tally register
8. Sterile test tubes (13 x 100 mm)

Media and reagents

1. Trypticase (tryptone) soy agar (TSA)
2. Baird-Parker medium
3. Sterile coagulase plasma (rabbit) with EDTA
4. Lysostaphin solution
5. Hydrogen peroxide (3%, v/v)
6. Toluidine blue-deoxyribonucleic (DNA) acid agar
7. 0.02 M phosphate-saline buffer containing 1% NaCl
8. Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate
9. Paraffin oil, sterile
10. Phenol Red Carbohydrate Broth

Sample preparation

Under aseptic conditions, prepare serial dilutions of sample by transferring 10 ml of previous dilution to 90 ml of diluent using separate pipets. Avoid sample foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 seconds.

Isolation

For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily adsorbed, place plates upright in incubator for about 1 h. Invert plates and incubate 45-48 h at 35°. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasionally from various foods

and dairy products, nonlipolytic strains of similar appearance may be encountered, except that surrounding opaque and clear zones are absent. Strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

Enumeration

Count and record colonies. If several types of colonies are observed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. If plates containing >200 colonies have colonies with the typical appearance of *S. aureus* and typical colonies do not appear at higher dilutions, use these plates for the enumeration of *S. aureus*, but do not count nontypical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

Identification of *S. aureus*

a. *Coagulase test*

Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml TSB containing 10% NaCl and 1% sodium pyruvate broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of TSB suspension. Incubate TSB culture suspension and slants 18-24 h at 35°. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA (B-4, above) to the TSB culture and mix thoroughly. Incubate at 35° and examine periodically over a 6 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further (for this method see: Sperber, W.H. and Tatini, S.R. 1975. Interpretation of the tube coagulase test for identification of *Staphylococcus aureus*. Appl. Microbiol. 29:502-505). Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically. A latex agglutination test (AUREUS TEST™, Trisum Corp., Taipei, Taiwan) may be substituted for the coagulase test if a more rapid procedure is desired.

Ancillary identification tests

a. *Catalase test*

On a clean glass slide or spot plate, emulsify growth from TSA slant in 3% hydrogen peroxide. Production of gas bubbles shows a positive reaction. Include known positive and negative cultures.

b. *Anaerobic utilization of glucose*

Inoculate tube of Phenol Red Carbohydrate broth containing glucose (0.5%). Immediately inoculate each tube heavily with wire loop. Make certain inoculum reaches bottom of tube. Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 35°. Acid is produced anaerobically if indicator

changes to yellow throughout tube, indicating presence of *S. aureus*. Run controls simultaneously (positive and negative cultures and medium controls).

c. *Anaerobic utilization of mannitol*

Repeat b, above, using mannitol as carbohydrate in medium. *S. aureus* is usually positive but some strains are negative. Run controls simultaneously.

d. *Lysostaphin sensitivity*

Transfer isolated colony from agar plate with inoculating loop to 0.2 ml phosphate-saline buffer, and emulsify. Transfer half of suspended cells to another tube (13 x 100 mm) and mix with 0.1 ml phosphate-saline buffer as control. Add 0.1 ml lysostaphin (dissolved in 100 ml of 0.02 M phosphate-saline buffer containing 1% NaCl for a final concentration of 25 µg lysostaphin/ml) to original tube. Incubate both tubes at 35° for not more than 2 h. If turbidity clears in test mixture, test is considered positive. If clearing has not occurred in 2 h, test is negative. *S. aureus* is generally positive.

e. *Thermostable nuclease production*

This test is claimed to be as specific as the coagulase test but less subjective, because it involves a color change from blue to bright pink. It is not a substitute for the coagulase test but rather is a supportive test, particularly for 2+ coagulase reactions. Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. When agar has solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate slides in moist chamber 4 h at 35°. Development of bright pink halo extending at least 1 mm from periphery of well indicates a positive reaction.

Characteristics

Some typical characteristics of 2 species of staphylococci and the micrococci, which may be helpful in their identification, are listed in Table 1.

Table 1. Typical characteristics of *S. aureus*, *S. epidermidis*, and micrococci[a]

Characteristic	*S. aureus*	*S. epidermidis*	Micrococci
Catalase activity	+	+	+
Coagulase production	+	-	-
Thermonuclease production	+	-	-
Lysostaphin sensitivity	+	+	-
Anaerobic utilization of			
glucose	+	+	-
mannitol	+	-	-

[a] +, Most (90% or more) strains are positive; -, most (90% or more) strains are negative.

MPN method

Note: *The most probable number (MPN) method is recommended for routine surveillance of products in which small numbers of S. aureus are expected and in foods expected to contain a large population of competing species.*

A. Equipment and materials - Same as for Direct Plate Count Method.
B. Media and reagents - Same as for Direct Plate Count Method. Also required, Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate.
C. Preparation of sample - Same as for Direct Plate Count Method.
D. Determination of MPN

Inoculate 3 tubes of TSB containing 10% NaCl and 1% sodium pyruvate with 1 ml portions of decimal dilutions of each sample. Highest dilution must give negative endpoint. Incubate tubes 48 ± 2 h at 35°. Using 3 mm loop, transfer 1 loopful from each tube showing growth (turbidity) to plate of Baird-Parker medium with properly dried surface. Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak inoculum to obtain isolated colonies. Incubate plates 48 h at 35°. From each plate showing growth, transfer at least 1 colony suspected to be *S. aureus* to TSB broth (see C of Direct Plate Count Method above). Continue procedure for identification and confirmation of *S. aureus* (see D of Direct Plate Count, above).

Media and reagents

<u>Media</u>

Baird-Parker Medium

Tryptone: 10 g
Beef extract: 5 g
Yeast extract: 1 g
Sodium pyruvate: 10 g
Glycine: 12 g
Lithium chloride $6H_2O$: 5 g
Agar: 20 g

Distilled water: 1 litre

Autoclave 15 min at 121°. Final pH, 7.0 ± 0.2. If desired for immediate use, maintain melted medium at 48-50° before adding enrichment. Otherwise, store solidified medium at 4 ± 1° up to 1 month. Melt medium before use.

Trypticase (Tryptic) Soy Agar

Trypticase peptone: 15 g
Phytone peptone: 5 g
NaCl: 5 g
Agar: 15 g

Distilled water: 1 litre

Heat with agitation to dissolve agar. Boil 1 min. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2.

Toluidine Blue-DNA Agar

Deoxyribonucleic acid (DNA): 0.3 g
Agar: 10 g
$CaCl_2$ (anhydrous): 1.1 mg
NaCl: 10 g
Toluidine blue O: 83 mg
Tris (hydroxymethyl) aminomethane: 6.1 g

Distilled water: 1 litre

Dissolve Tris (hydroxymethyl) aminomethane in 1 liter distilled water. Adjust pH to 9.0. Add the remaining ingredients except toluidine blue O and heat to boiling to dissolve. Dissolve toluidine blue O in medium. Dispense to rubber-stoppered flasks. Sterilization is not necessary if used immediately. The sterile medium is stable at room temperature for 4 months and is satisfactory after several melting cycles.

Reagents

Lysostaphin solution

Dissolve 2.5 mg of lysostaphin in 100 ml of 0.02M phosphate-saline buffer containing 1% NaCl to a final lysostaphin concentration of 25 µg/ml.

0.02 M Phosphate Buffer (pH 7.3-7.4)

Stock Solution 1

Sodium phosphate dibasic anhydrous: 28.4 g
Na_2HPO_4 (anhydrous; reagent grade)

NaCl: 85 g

Distilled water: 1 litre

Stock Solution 2

Sodium phosphate monobasic monohydrate: 27.6 g
$NaH_2PO_4.H_2O$ (monohydrate; reagent grade)

NaCl: 85 g

Distilled water: 1 litre

To obtain 0.02 M phosphate-buffered saline (0.85%), make 1:10 dilutions of each stock solution. For example:

Stock solution 1	50 ml	Stock solution 2	10 ml
Distilled water	450 ml	Distilled water	90 ml
Approximate pH, 8.2		Approximate pH, 5.6	

Using a pH meter, titer diluted solution 1 to pH 7.3-7.4 by adding about 65 ml of diluted solution 2. Use resulting 0.02 M phosphate saline buffer solution in the lysostaphin susceptibility test on *S. aureus*.

Note: Do not titer 0.2 M phosphate buffer to pH 7.3-7.4 and then dilute to 0.02 M strength. This results in a drop in pH of approximately 0.25. Addition of 0.85% salt after pH adjustment also results in a drop of approximately 0.2.

Annex 1: Summary of recommendations from the 67th JECFA and further information required

Toxicological recommendations and information on specifications

1. Food additives and ingredients evaluated toxicologically or assessed for dietary exposure

Food additive	Specifi-cations[a]	Acceptable daily intake (ADI) and other toxicological recommendations
Annatto Extracts	R	**ADI for bixin of 0-12 mg/kg bw** Applicable to the following Annatto extracts, provided they comply with the respective specifications: - solvent-extracted bixin (≥85 % bixin, ≤2.5% norbixin) - aqueous processed bixin (≥25 % bixin, ≤7% norbixin) Does not apply to oil-processed bixin (≥10 % bixin) **Group ADI for norbixin and its sodium and potassium salts of 0-0.6 mg/kg bw (expressed as norbixin)** Applicable to the following Annatto extracts, provided they comply with the respective specification: - solvent extracted norbixin (≥85 % norbixin) - alkali processed norbixin, acid precipitated (≥35% norbixin) and not acid precipitated (≥15 % norbixin) In re-evaluating the studies of toxicity with solvent-extracted bixin (92% bixin) and solvent-extracted norbixin (91.6% norbixin) and in light of the additional compositional data, the Committee considered that ADIs could be allocated to these pigments, based on the studies conducted on the extracts. The Committee established an ADI for bixin of 0–12 mg/kg bw on the basis of the NOEL of 1311 mg/kg bw per day from a 90-day study in male rats fed an extract containing 92% bixin, corrected for pigment content and applying a safety factor of 100. The Committee established a group ADI for norbixin and its sodium and potassium salts of 0–0.6 mg/kg bw (expressed as norbixin) on the basis of the NOEL of 69 mg/kg bw per day from a 90-day study in male rats fed an extract containing 91.6% norbixin, corrected for pigment content and applying a safety factor of 100. Based on compositional data and toxicological data on aqueous processed bixin and alkali-processed norbixin (acid precipitated), the Committee concluded that the use of these annatto extracts as sources of bixin or norbixin would not raise safety concerns, provided that they complied with the relevant specifications. Accordingly, the ADIs given above could be applied to bixin and norbixin derived from these annatto extracts. The Committee noted that the pigment in alkali-processed norbixin (not acid-precipitated) consists of sodium or potassium salts of norbixin and that compositional data on this extract, complying with the specifications, did not raise safety concerns. Consequently, the Committee concluded that the group ADI for norbixin and its sodium and potassium salts is applicable to norbixin salts from this source. As no NOEL could be identified for oil-processed bixin and no compositional data were available, the Committee decided that the above evaluation could not be applied to this extract.

		Assuming all annatto derived pigment were bixin, the estimated intake would amount to approximately 0.2% of the ADI (0–12 mg/kg bw). Assuming all annatto derived pigment were norbixin, the estimated intake would amount to approximately 4% of the ADI (0–0.6 mg/kg bw). Specifications have been established for all extracts which are covered by the established ADIs, and tentative specifications for oil-processed bixin.
Lycopene (synthetic)	N	The Committee established an **ADI of 0–0.5 mg/kg bw for synthetic lycopene** based on the highest dose of 50 mg/kg bw per day tested in the 104-week study in rats (at which no adverse effects relevant to humans were induced), and a safety factor of 100. **This ADI was made into a group ADI to include lycopene from *Blakeslea trispora*,** which was also under consideration at the present meeting and was considered to be toxicologically equivalent to chemically synthesized lycopene. The estimate of high exposure (greater than 95th percentile) of 30 mg/person per day, equivalent to 0.5 mg/kg bw per day, which includes background exposure plus additional exposure from food additive uses, is compatible with the ADI.
Lycopene from *Blakeslea trispora*	N	Lycopene from *Blakeslea trispora* is considered to be toxicologically equivalent to chemically synthesized lycopene, for which an ADI of 0–0.5 mg/kg bw was established. This was given further credence by the negative results obtained for lycopene from *B. trispora* in two tests for genotoxicity, and the absence of adverse effects in a short-term toxicity study considered at the present meeting. **The ADI for synthetic lycopene was therefore made into a group ADI of 0-0.5 mg/kg bw to include lycopene from *B. trispora*.** The exposure estimate is the same as for synthetic lycopene.
Natamycin (aka pimaricin) (exposure assessment)		The data as a whole, including estimations based on GEMS/Food Consumption Cluster Diets and calculations for consumers with a high intake and children, confirm the results of the assessment made by the Committee at its fifty-seventh meeting and show that the **current ADI of 0–0.3 mg/kg bw is unlikely to be exceeded.**
Propyl paraben (aka propyl para-hydroxybenzoate)	W	In view of the adverse effects in male rats, **propyl paraben (propyl p-hydroxybenzoate) should be excluded from the group ADI for the parabens used in food.** This conclusion was reached on the grounds that the group ADI was originally set on a NOEL of 1000 mg/kg bw per day for a different toxicological end-point—growth depression—taken from the range of studies then available for the methyl, ethyl and propyl parabens. Propyl paraben has shown adverse effects in tissues of reproductive organs in male rats at dietary doses of down to 10 mg/kg bw per day, which is within the range of the group ADI (0–10 mg/kg bw), with no NOEL yet identified. The specifications for propyl paraben were withdrawn. **The group ADI of 0–10 mg/kg bw for the sum of methyl and ethyl esters of p-hydroxybenzoic acid was maintained.**

[a] N: new specifications prepared; R: existing specifications revised; W: specifications withdrawn

2. Food additives considered for specifications only

Food Additive	Specifications[a]
Acetylated oxidized starch	R
Annatto extracts (oil processed bixin)	R, T
Butyl p-hydroxybenzoate (butyl paraben)	W
Carob bean gum	R, T
Carob bean gum (clarified)	N, T
Ethylene oxide	W
Guar gum	R, T
Guar gum (clarified)	N, T
DL-Malic acid and its calcium and sodium salts	R
Maltitol	R
Titanium dioxide	R
Zeaxanthin (synthetic)	R

[a]N: new specifications prepared; R: existing specifications revised; T: tentative specifications; W: specifications withdrawn.

3. Food contaminants evaluated toxicologically

Food Contaminant	Tolerable intakes and other toxicological recommendations
Aluminium (from all sources including food additives)	The Committee established **a PTWI for Al of 1 mg/kg bw**, which applies to all aluminium compounds in food, including additives. The previously established ADIs and PTWI for aluminium compounds were withdrawn. The Committee concluded that aluminium compounds have the potential to affect the reproductive system and developing nervous system at doses lower than those used in establishing the previous PTWI and therefore revised the PTWI. The available studies have many limitations and are not adequate for defining the dose–response relationships. The Committee therefore based its evaluation on the combined evidence from several studies. The relevance of studies involving administration of aluminium compounds by gavage was unclear because the toxicokinetics following gavage were expected to differ from toxicokinetics following dietary administration, and these gavage studies generally did not report total aluminium exposure including basal levels in the feed. The studies conducted with dietary administration of aluminium compounds were considered most appropriate for the evaluation. The lowest LOELs for Al in a range of different dietary studies in mice, rats and dogs were in the range of 50–75 mg/kg bw per day. The Committee applied an uncertainty factor of 100 to the lower end of this range of LOELs (50 mg Al/kg bw per day) to allow for inter- and intra-species differences. There are deficiencies in the database, notably the absence of NOELs in the majority of the studies evaluated and the absence of long-term studies on the relevant toxicological end-points. These deficiencies are counterbalanced by the probable lower bioavailability of the less soluble aluminium compounds present in food. Overall, it was considered appropriate to apply an additional uncertainty factor of 3. The Committee confirmed that the resulting health-based guidance value should be expressed as a PTWI, because of the potential for bioaccumulation. The Committee noted that the PTWI is likely to be exceeded to a large extent by some population groups, particularly children, who regularly consume foods that include aluminium-containing additives. The Committee also noted that dietary exposure to Al is expected to be very high for infants fed on soya-based formula.
3-chloro-1,2-propanediol	As no new pivotal toxicological studies had become available the Committee **retained the previously established PMTDI of 2 µg/kg bw** for 3-chloro-1,2-propanediol. Estimated exposures at the national level considered a wide range of foods, including soy sauce and soy-sauce related products, ranged from 1% to 35% of the PMTDI for average exposure in the general population. For the consumers at the high percentile (95th), the estimated intakes ranged from 3% to 85% and up to 115% of the PMTDI in young children. These estimates are based on concentrations of 3-chloro-1,2-propanediol derived before any remedial action had been taken by government or industry. The Committee noted that reduction in the concentration of 3-chloro-1,2-propanediol in soy sauce and related products made with acid-HVP could substantially reduce the intake of this contaminant by certain consumers of this condiment.

1,3-dichloro-2-propanol	The Committee concluded that the critical effect of 1,3-dichloro-2-propanol is carcinogenicity. The substance yielded negative results in two new studies on genotoxicity in vivo, but limitations in these studies and positive findings in tests for genotoxicity in vitro as well as lack of knowledge on the modes of action operative at the various tumour locations led the Committee to the conclusion that a genotoxic mode of action could not be excluded. Accordingly, the cancer dose–response data were analysed by dose–response modelling to calculate BMD10 and BMDL10 values. The Committee concluded that a representative mean intake for the general population of 1,3-dichloro-2-propanol of 0.051 μg/kg bw per day and an estimated high-level intake (young children included) of 0.136 μg/kg bw per day could be used in the evaluation. Comparison of these mean and high-levels intakes with the lowest BMDL10 of 3.3 mg/kg bw per day, which was the BMDL10 for incidence data on tumour-bearing animals for all treatment-affected locations, indicates **margins of exposure of approximately 65 000 and 24 000, respectively. Based on these margins of exposure, the Committee concluded that the estimated intakes of 1,3-dichloro-2-propanol were of low concern for human health.** The available evidence suggests that 1,3-dichloro-2-propanol occurs at lower levels than 3-chloro-1,2-propanediol in soy sauce and related products, and also in acid-HVP food ingredients. However, in meat products the concentrations of 1,3-dichloro-2-propanol are generally higher than the levels of 3-chloro-1,2-propanediol.
Methylmercury	The Committee made it clear that the previous PTWI of 3.3 μg/kg bw had, in fact, been withdrawn in 2003. The Committee **confirmed the existing PTWI of 1.6 μg/kg** bw, set in 2003, based on the most sensitive toxicological end-point (developmental neurotoxicity) in the most susceptible species (humans). However, the Committee noted that **life-stages other than the embryo and fetus may be less sensitive to the adverse effects of methyl mercury.** In the case of adults, the Committee considered that intakes of up to about two times higher than the existing PTWI of 1.6 μg/kg bw would not pose any risk of neurotoxicity in adults, although in the case of women of childbearing age, it should be borne in mind that intake should not exceed the PTWI, in order to protect the embryo and fetus. Concerning infants and children aged up to about 17 years, the data do not allow firm conclusions to be drawn regarding their sensitivity compared to that of adults. While it is clear that they are not more sensitive than the embryo or fetus, they may be more sensitive than adults because significant development of the brain continues in infancy and childhood. Therefore, the Committee could not identify a level of intake higher than the existing PTWI that would not pose a risk of developmental neurotoxicity for infants and children. The Committee has previously noted that fish makes an important contribution to nutrition, especially in certain regional and ethnic diets. The present Committee recommends that the known benefits of fish consumption need to be taken into consideration in any advice aimed at different subpopulations. Risk managers may wish to consider whether specific advice should be given concerning children and adults, after weighing the potential risks and benefits. The Committee concluded that the setting of guideline levels for methyl mercury in fish may not be an effective way of reducing exposure for the general population. The Committee noted that advice targeted at population subgroups that may be at risk from methyl mercury exposure may provide an effective method for lowering the number of individuals with exposures greater than the PTWI.

Further information required

Annatto extracts (oil-processed bixin)
Information is required on the chemical characterisation of the non-colouring matter components of commercial products. The tentative specifications monograph will be withdrawn unless the requested information is received before the end of 2008.

Carob bean gum
Data are required on gum content, solubility in water and an analytical method using capillary gas chromatography for measuring residual solvents. For clarified carob bean gum, in addition to the information listed above for carob bean gum, information is requested on synonyms and a range of other information on purity. The tentative specifications monograph will be withdrawn unless the required information is received before the end of 2007.

Guar gum
Data are required on gum content and an analytical method using capillary gas chromatography for measuring residual solvents. For clarified guar gum, in addition to the information listed above for guar gum, information is requested on synonyms and a range of other information on purity. The tentative specifications monograph will be withdrawn unless the required information is received before the end of 2007.

Aluminium
Further data on the bioavailability of different aluminium-containing food additives are required. There is a need for an appropriate study of developmental toxicity and a multigeneration study incorporating neurobehavioural end-points, to be conducted on a relevant aluminium compound(s). Studies to identify the forms of aluminium present in soya formulae, and their bioavailability, are needed before an evaluation of the potential risk for infants fed on soya formulae can be considered.

3-chloro-1,2-propanediol
The Committee noted that it has been reported that fatty acid esters of 3-chloro-1,2-propanediol are present in foods, but there were insufficient data to enable either their intake or toxicological significance to be evaluated. The Committee recommended that studies be undertaken to address this question.

CORRIGENDUM

COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS
FAO FOOD AND NUTRITION PAPER 52, Addendum 13, ROME, 2005.

Page 2, second heading ***Monomagnesium phosphate and trisodium phosphate*** is replaced by ***Monomagnesium phosphate and trisodium diphosphate***

Page 65, flavouring agent No. 1572 is listed with incorrect chemical name cis-Carvone-5,6-oxide and synonym cis-Carvone oxide. The name is replaced by trans-Carvone-5,6-oxide and the synonym by trans-Carvone oxide.

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COMPENDIUM OF FOOD ADDITIVE SPECIFICATIONS

Joint FAO/WHO Expert Committee on Food Additives

67th meeting 2006

This document contains food additive specifications monographs, analytical methods and other information, prepared at the sixty-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which was held in Rome, Italy, from 20 to 29 June 2006. The specifications monographs provide information on the identity and purity of food additives used directly in foods or in food production. The main three objectives of these specifications are to identify the food additive that has been subjected to testing for safety, to ensure that the additive is of the quality required for use in food or in processing, and to reflect and encourage good manufacturing practice. This publication and other documents produced by JECFA contain information that is useful to all those who work with or are interested in food additives and their safe use in food.

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